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Proteomic Analysis of *Bacillus cereus* Spores

K. E. Schwandt and B. J. Berger
Defence R&D Canada – Suffield

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Abstract

Spores were harvested from *Bacillus cereus* 14579 grown in casitone-yeast medium for 4 days at 30°C. Two-dimensional gel electrophoresis was performed on solubilized spore protein and twenty of the most abundant spots were analyzed by N-terminal amino acid sequencing. The identities of the N-terminal sequences obtained were determined by homology searching of the Genbank non-redundant database, and the *B. anthracis* and *B. cereus* genome projects. All of the identified proteins were plausible spore components, and included chaperonins, sporulation regulators, ribosomal proteins, proteases, and metabolic enzymes involved in energy production, radical detoxification, and germination. The conservation and extremely high identity of the identified proteins in both the *B. cereus* and *B. anthracis* genome databases confirmed the applicability of *B. cereus* spores as a surrogate model for the study of *B. anthracis* spores.

Résumé

Des spores ont été récoltées du *Bacillus cereus* 14579 lui-même cultivé dans un médium de levure et de casitone pendant une durée de 4 jours à une température de 30°C. L'électrophorèse bidimensionnelle en gélose a été effectuée sur des protéines de spores solubilisées et vingt des pores les plus abondantes ont été analysées par un séquençage aminoacide N-terminal. L'identité des séquences N-terminal obtenue a été déterminée par une recherche d'homologie dans la base de données Genbank non redondante et les projets de génomes *B. anthracis* et *B. cereus*. Toutes les protéines identifiées étaient des composantes crédibles et comprenaient des chaperonines, des protéases, des transporteurs ainsi que des enzymes participant à la production d'énergie, la détoxification de radicaux et la germination. La conservation et la très importante correspondance de protéines identifiées dans les bases de données de génomes de *B. cereus* et *B. anthracis* confirment les possibilités d'application des spores *B. cereus* comme modèle de substitution, pour l'étude des spores *B. anthracis*.

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Executive summary

Given the the recent cases of anthrax in the United States due to dissemination of bacterial spores via the mail, there is an increased interest in issues relating to the detection and inactivation of anthrax spores. Hampering the development of solutions to these problems is the relative lack of knowledge of the proteinaceous constitutents of the spore itself, and the very high level of pathogenicity of *Bacillus anthracis*. The aim of this initial study on spore proteins was two-fold: to demonstrate that *Bacillus cereus* can act as a highly accurate model for the determination of anthrax spore constituents, and to identify a number of the most abundant proteins present in the spore.

B. cereus spores were cultured and isolated with the same strain of the bacterium used for the recently available genome database for the organism. The spores were then treated with a strong denaturing buffer in order to solubilise most, but not all, of the protein present. The protein mixture was then separated first by charge and then by size using two-dimensional gel electrophoresis, and individual protein spots excised. Each spot was then subjected to N-terminal amino acid sequencing to provide information on the exact sequence of one end of each protein. The resulting N-terminal peptide sequence was then used as an input for homology searching of the *B. cereus* and *B. anthracis* genome databases, as well as the all-inclusive Genbank non-redundant database. The consensus of the best matches for each database was used to identify each protein sequence.

The matching sequences obtained from the *B. cereus* and *B. anthracis* genome data were nearly identical, highlighting the extremely close relationship between these organisms at the genetic level. In addition, the complete conservation of the identified sequences demonstrated that *B. cereus* can act as a convenient, low pathogenicity model for anthrax in the examination of spore components.

All the proteins identified from the *B. cereus* spore preparations appeared to be plausible spore components. All of the proteins identified appeared to have functional, rather than structural, roles in the spore, and included ribosomal proteins, chaperonins, proteases, transporters, and enzymes involved in energy production, radical detoxification, and germination. Several proteins, such as alanine racemase, leucine dehydrogenase, camelysin, and GroEL have been previously identified as spore components in *B. cereus*, *B. anthracis*, or *B. subtilis*. The rediscovery of these proteins in this study provides confidence that the present approach to spore characterisation yields results consistent with existing data.

Direct knowledge of the exact constituents of the bacterial spore represents the first step in the design of better diagnostic tests and agents capable of deactivating the spore or preventing germination. A number of the proteins uncovered in the present study represent intriguing targets for further diagnostic or therapeutic examination.

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Sommaire

Les cas récents de charbon bactéridien aux États-Unis dus à la dissémination des spores bactériennes dans les services postaux, ont suscité un nouvel intérêt au sujet des problèmes de détection et d'inactivation des spores de charbon. La mise au point de solutions à ce problème a été retardée par un certain manque de connaissance des constituants protéiques de la spore elle-même et du très haut niveau de pathogénicité du *Bacillus anthracis*. Le but de cette étude initiale des protéines de spores comporte deux aspects: il s'agit de démontrer que le *Bacillus cereus* peut agir comme modèle exact dans la détermination des constituants de spores de charbon et aussi d'identifier un certain nombre de protéines comme étant les plus abondantes dans la spore.

Les spores B. *cereus* ont été cultivées et isolées avec la même souche de bactérie que celle utilisée par la base de données de génomes récemment mise à la disposition de l'organisme. Les spores ont été ensuite traitées avec un tampon dénaturant afin de solubiliser la plupart, mais pas toutes, des protéines présentes. Le mélange de protéines a été ensuite séparé d'abord en fonction de la charge puis de la taille en utilisant l'électrophèse bidimensionnelle en gélose et les taches de protéines individuelles ont été excisées. Chaque tache a été ensuite soumise au séquençage aminoacide N-terminal qui renseigne sur la séquence exacte d'une extrémité de chaque protéine. La séquence N-terminal de peptides a été ensuite utilisée comme entrée de données pour la recherche d'homologie des bases de données des génomes *B. cereus* et du *B. anthracis* ainsi que pour la base de données pluraliste et non redondante Genbank. Les meilleurs jumelages choisis par consensus dans chaque base de données ont été utilisés pour déterminer chaque séquence de protéines.

Les séquences de jumelage obtenues à partir des données de génomes *B. cereus* et *B. anthracis* sont presque identiques et mettent en relief la forte ressemblance de ces deux organismes au niveau génétique. De plus, la conservation complète des séquences identifiées indique que le *B. cereus* peut agir de modèle pratique de faible pathogénicité pour le charbon durant l'étude des composants de spores.

Toutes les protéines identifiées à partir des préparations de spores *B. cereus* apparaissent être des composants de spores crédibles. Toutes les protéines identifiées semblent avoir des rôles fonctionnels plutôt que structurels dans la spore; elles incluent les protéines ribosomiques, les chaperonines, les protéases, les transporteurs ainsi que les enzymes participant à la production d'énergie, la détoxification des radicaux et la germination. Plusieurs protéines telles que la racemase d'alanine, la déhydrogénase de leucine, le camelysin et le GroEL ont été identifiées précédemment comme composants de pores dans le *B. cereus*, *B. anthracis*, ou *B. subtilis*. La redécouverte de ces protéines dans cette étude permet de penser que la méthode actuelle de caractérisation des spores obtient des résultats qui sont compatibles avec les données déjà existantes.

La connaissance directe des constituants exacts des spores bactériennes représente la première étape de la conception de meilleurs tests de diagnostic et d'agents capables de désactiver la spore ou d'empêcher la germination. Un certain nombre de protéines révélées dans cette étude représentent des cibles fascinantes de diagnostics futurs ou d'examen thérapeutique.

Schwandt, K.E., and Berger, B.J. 2002. Proteomic analysis of *Bacillus cereus* spores. Defence R&D Canada – Suffield. TR2002-149.

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Acknowledgements

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The gapped *Bacillus cereus* genome project was made available by Integrated Genomics (www.integratedgenomics.com) and was funded by DARPA. The complete *Bacillus anthracis* genome was made available before publication by The Institute for Genomic Research (www.tigr.org) and was funded by DARPA.

Introduction

Since 1877, when Robert Koch grew the bacterium in pure culture, *Bacillus anthracis* has been known as the aetiological agent of anthrax. This organism was the factor behind the massive loss of livestock in Europe in the 1800's and still causes widespread death and disease in animals of the Third World [1] as well as Wood Buffalo National Park, Alberta [2]. Since its discovery, anthrax has been identified as a possible agent of biological warfare and this threat has secured anthrax a position at the forefront of defence research. Recently, letters containing anthrax spores were delivered in the Eastern United States. The spores contained within these letters caused the death of five people. These recent attacks, combined with an incomplete understanding of the pathogenesis and biochemistry of anthrax, highlight the need for additional research on the organism.

An excellent biological model for anthrax is *Bacillus cereus*. *B. cereus* is a food-borne opportunistic pathogen [3] responsible for some common types of food poisoning and is identical to *B. anthracis* except in its pathogenicity [4]. In fact, *Bacillus cereus*, *Bacillus anthracis*, and *Bacillus thuringiensis* are the same species differing only in plasmid content [5]. For these reasons, *B. cereus* can be used in studies to further the understanding of anthrax.

The spore is one of nature's most resilient and efficient means of preserving genetic information. In fact, a bacterial spore, closely related to *Bacillus sphaericus*, was revived, cultured, and identified from the abdominal contents of extinct bees preserved in ancient Dominican amber [6]. In addition, they contain factors that regulate germination to ensure that environmental conditions are optimal for growth [1]. Both *B. cereus* and *B. anthracis* are known to produce spores which invariably add to their resilience and biological persistence.

However, surprisingly little is actually known about the constituents of spores themselves. Questions still remain as to exactly what these are composed of, what is contained within, and the specific biochemistry. Two recent papers have emerged on the subject, one dealing with the constituents of the exosporium of *B. cereus* spores [4] and the other a study of heat stress response of *B. subtilis* during sporulation [7]. In regards to the first study, several proteins making up the exosporium were identified while the function of the exosporium remains unknown. Some of the proteins described in this report include Immune Inhibitor A, GroEL, and pyrroline-5-carboxylate dehydrogenase [4]. The presence of these proteins is unlikely to be of integral structural importance but rather suggests that the exosporium may have functional significance [4]. The second paper discusses the expression of new proteins during sporulation which are specific to heat shock such as DnaK, GroEL, DnaJ, GrpE, and GroES [7]. This study allowed for a better understanding of the resistance mechanisms of *Bacillus* spores to high temperatures.

The completion of the *B. subtilis* genome, and the recent momentum towards the completion of the *B. anthracis* and *B. cereus* genomes, have given rise to a defined data set containing potential spore proteins. For these reasons, a study was conducted into identifying the proteins contained within, or associated with, spores of *Bacillus cereus*. Through two-dimensional gel electrophoresis and N-terminal amino acid sequencing, a number of spore constiuents were identified. Although the information uncovered was broad, the methods devised and the information compiled should provide a starting point for future studies on the detection and inactivation of *Bacillus* spores.

Materials and Methods

Bacterial Strains and Culture Conditions

Bacillus cereus ATCC14579 was acquired from the American Type Culture Collection (Manassas, VA, USA). Unless otherwise stated all chemicals were obtained from Sigma Chemical Company (Oakville, ON, Canada). The *B. cereus* culture was grown in CCY broth (1.0 g casamino acid (Becton Dickinson and Company, Sparks, MD, USA), 1.0 g casitone (Becton Dickinson), 0.4 g yeast extract (Becton Dickinson), 13 mM KH_2PO_4, 26 mM K_2HPO_4, 20 mg L-glutamine, 0.5 mM $MgCl_2$-$6H_2O$, 0.01 mM $MnCl_2$-$4H_2O$, 0.05 mM $FeCl_3$-$6H_2O$, 0.05 mM $ZnCl_2$, 0.2 mM $CaCl_2$-$6H_2O$, 0.48 mL glycerol, all dissolved in 1.0 L distilled deionized water [ddH_2O]). Cultures were incubated in a controlled environment incubator shaker (New Brunswick Scientific Company Inc., Edison, NJ, USA) at 30°C and 250 rotations per minute (rpm) for 4 days.

The extent of sporulation in cultures was determined with a Neubauer hemocytometer under phase contrast microscopy. After spore harvesting, a slide preparation of the spore suspension was heat fixed and spore stained with Malachite Green (Difco Laboratories, Detroit, Michigan, USA) and Safranin O (Allied Chemical & Dye Corporation, New York City, NY, USA).

Spore Harvesting

When greater than 90% free spores were present in the culture the spores were harvested by centrifugation with a Beckman J2-MI centrifuge (Beckman Instruments Inc., Mississauga, ON, Canada) and a JA-10 rotor at 11°C and 7500 rpm. The supernatant was decanted and the pellets were rinsed with a small amount of ddH_2O to remove cellular debris and unsporulated cells. The pellet was then resuspended in ddH_2O and centrifuged a second time at 8°C and 7500 rpm. Again the liquid was decanted, the pellet was resuspended in ddH_2O, and centrifuged a final time at 4°C and 7500 rpm. The supernatant was poured off and the pellet was resuspended in 5 mL of distilled water. This suspension was vortexed to completely resuspend the pellet and then aliquoted in 500 µL amounts into microtubes which were stored at -70°C.

Preparation of Protein Samples

A protein suspension was prepared by sonication of a 500 µL spore preparation with a Soniprep 150 probe sonicator (MSE, Markham, ON, Canada) and then lyophilisation in an Eppendorf vacufuge (Brinkmann Instruments, Mississauga, ON, Canada). The dried homogenate was then solubilized in 500 µL rehydration buffer (8 M urea, 2 M thiourea, 1% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate dihydrate [CHAPS] (Aldrich Chemical Company, Oakville, ON, Canada), 20 mM dithiothreitol [DTT], 0.2% Bio-Lyte 3/10 Ampholytes (Bio-Rad Laboratories, Mississauga, ON, Canada), and bromothymol blue, dissolved in 10 mL of ddH_2O) and centrifuged for 10 minutes at 10000 rpm in an Eppendorf Centrifuge 5417 C (Brinkmann Instruments).

Two-dimensional Gel Electrophoresis

The proteins present in the *B. cereus* spores were resolved on 7 cm Immobilised pH Gradient (Ready Strip IPG) strips with a pH range from pH 3 to 10 (Bio-Rad). 200 µL of solubilized protein sample was placed on the IPG strip, and then 800 µL of isoelectric focussing (IEF) mineral oil (Bio-Rad) was pipetted over the strip. Gel rehydration consisted of an active rehydration program of 50 V applied over a minimum of 12 hours in a Protean IEF cell (BioRad). Focussing then took place at 20°C and 250 V for 15 minutes, followed by a linear increase to 8000 V over 2 hours. The system then remained at 8000 V for 20000 volt hours when it dropped to a 500 V hold.

The IPG strip was then placed in 1 mL of equilibration buffer (6 M urea, 2% (w/v) sodium dodecyl sulfate [SDS], 0.375 M Trizma Base titrated with HCl to pH 8.8, 20% glycerol, and 130 mM DTT, dissolved in 10 mL ddH_2O) for 15 minutes followed by another 15 minutes in 1 m of neutralisation buffer (6 M urea, 2% (w/v) SDS, 0.375 M Trizma Base titrated with HCl to pH 8.8, 20% glycerol, and 135 mM iodoacetamide, dissolved in 10 mL ddH_2O).

The IPG strip was then inserted into the well of a 10% SDS polyacrylamide gel and sealed with 1% agarose and bromothymol blue in 1X SDS-PAGE running buffer (0.30 g Trizma Base, 1.44 g glycine, 0.10 g SDS in 1.0 L ddH_2O). For this electrophoresis step the Bio-Rad Mini-Protean electrophoresis apparatus was used with 1X running buffer and a Bio-Rad Model 300Xi computer controlled power supply set at a constant current of 13 mA. After electrophoresis, the gel was stained with Coomassie blue R250, and photographed.

Transblotting Spore Proteins on PVDF

The two-dimensional gel was electroblotted onto polyvinylidene difluoride [Sequi-Blot PVDF membrane] (Bio-Rad) using the Bio-Rad Mini-Protean transblotting apparatus filled with blotting buffer (10 mM Trizma Base, 100 mM glycine, and 10% methanol (EM Science, Gibbstown, NJ, USA) in 1.0 L of ddH_2O). The Bio-Rad Model 300Xi computer controlled power supply was used set for 1 hour at 100 V. The PVDF membrane was then washed ten times in ddH_2O for 5 minutes each and stained with 0.1% Amido Black in 10% glacial acetic acid (BDH Inc., Toronto, ON, Canada) for one minute. Finally, the membrane was destained with 5% glacial acetic acid for one minute and then rinsed twice for 10 minutes in ddH_2O and photographed. The desired spots were then excised, lyophilisised, and stored individually in microtubes at 4°C.

N-terminal Amino Acid Sequencing and Sequence Analysis

Each excised spot was sequenced on the Applied Biosystems, Procise Protein Sequencing System (Applied Biosystems, Foster City, CA, USA) for 20 cycles under the pulsed-liquid setting. The sequences called by this system were identified by searching Genbank, a gapped *B. cereus* genome data set, or a complete *B. anthracis* genome set using the BLAST program [8]. Local BLAST searching of *Bacillus* databases and graphical presentation of protein alignments was accomplished using the BioEdit program (Hall, 1999).

The gapped *B. cereus* genome data was made available by Integrated Genomics (www.integratedgenomics.com), and the complete *B. anthracis* genome data was made available before publication by The Institute for Genomic Research (www.tigr.org) and was subsequently published by Read et al. [9].

Results and Discussion

Assessment of Spore Purification

A heat fixed smear of the harvested spore preparation from CCY broth was stained for spores. With this method spores took up Malachite Green while vegetative cells and debris took up Safranin. Assessment of the slide revealed greater than 93% free spores.

Method Adjustments

Optimizing solubilization and isoelectric focusing of the spore samples involved several adjustments from the conditions recommended by BioRad for IPG strips. First, 2 M of thiourea and 0.2% ampholytes were added to the rehydration buffer. Thiourea greatly increased the degree of sample solubilisation. Dropping the ampholytes from 2% to 0.2% decreased sample resistance and allowed for higher running voltages. Secondly, the isoelectric focussing program was modified from a 4000 V linear increase, to an 8000V linear increase creating tighter isoelectric focussing on the IPG strips.

Identification of Proteins

Approximately 152 spots were resolved on the 2D gel (Figure 1). Twenty of the most abundant spots were selected and excised for N-terminal sequencing (Figure 2). From these twenty spots, twenty-five protein sequences were identified from database searches described in the Materials and Methods (Table 1). The range of molecular weight and pI values for each spot in the 2D gels matched well with the theoretical weight and pI values of the identified proteins found in the *B. cereus* and *B. anthracis* genome databases (Table 2).

The amino acid sequence found in Spot 1 was identified as translation elongation factor G (EF-G) based on matches with *B. cereus*, *B. anthracis* and *B. halodurans* sequences (Table 1 and Figure 3). The identity of spot 1 was also confirmed by comparison of the calculated ranges of the excised spot with the molecular weight and pI of EF-G found in *B. anthracis* (Table 2). The molecular weight of spot 1 was a good match for *B. anthracis* EF-G while the pI was slightly more basic. Since the c-terminus of *B. cereus* fell within a gap in the genome an accurate molecular weigh and pI were available for comparison. Elongation factors are GTPases that are crucial for protein synthesis [10]. These enzymes catalyze the translocation step, coupled with breakdown of GTP, causing the movement of mRNA and its combined tRNA's in relation to the ribosome. The GPTase activity of EF-G in particular is stimulated by ribosomal protein L7/12 [10]. Since protein synthesis is fundamental to germination of the spore the presence of this protein in the spore is unsurprising.

The amino acid sequence from spot 2 was identified as dnaK from the heat shock protein 70 (hsp70) family. The sequence matched closely with hsp70 *of B. cereus*, *B. anthracis* and *Streptococcus agalactiae* (Table1 and Figure 4). The range of molecular weight and pI for spot 2 put it just slightly heavier and somewhat less acidic than *B. anthracis* hsp70 (Table 2). Again in this case the C-terminus of *B. cereus* hsp70 fell in a gap in the genome data, and therefore correct molecular weights and pI values could not be calculated for its sequence.

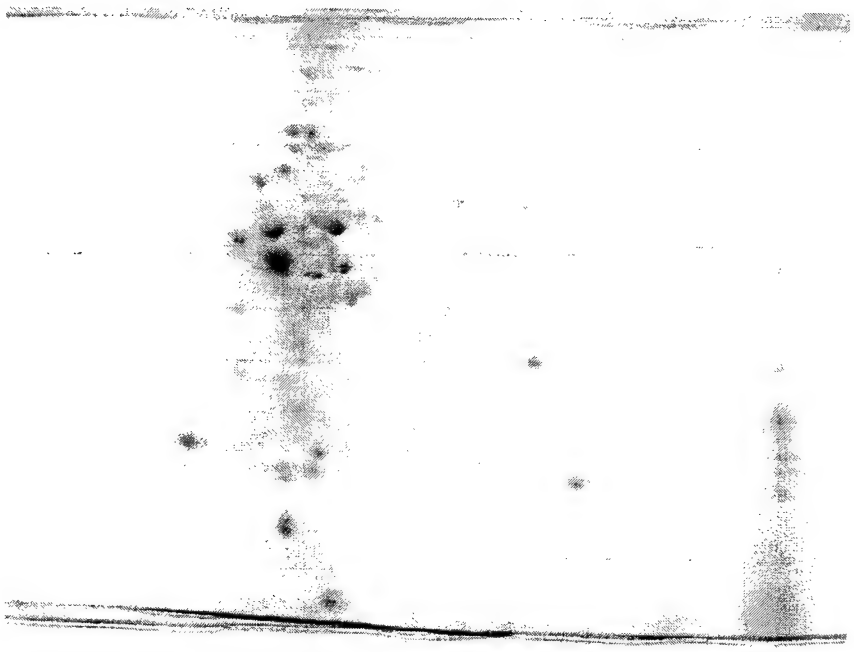

Figure 1. *Two-dimensional gel of B. cereus spore proteins. Solubilised B. cereus proteins were separated on an immobilised polyacrylamide gel strip (pH 3 – 10, left to right), followed by separation on a 10% SDS polyacrylamide gel (top to bottom).*

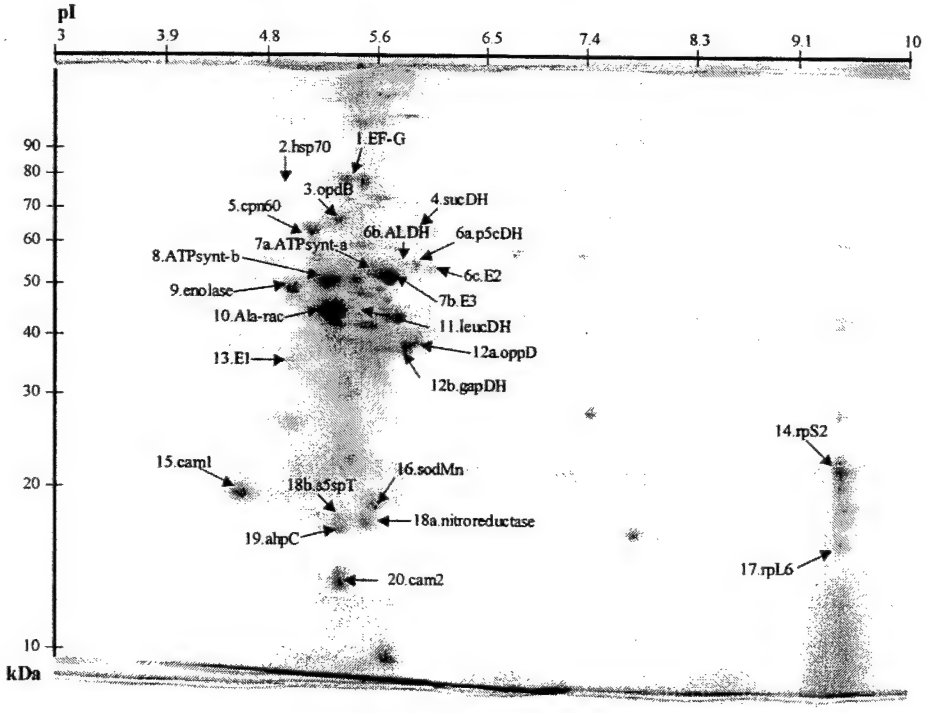

Figure 2. *Labelled spots on a two-dimensional gel of B. cereus spore proteins.*

Table 1. *Protein identities from 2D gels of B. cereus spore proteins.*

SPOT #	N-TERMINAL SEQUENCE	*B. cereus* MATCH[a]		*B. anthracis* MATCH[b]		BLAST MATCH[c]	
		Accession #	*Identity*	*Accession #*	*Identity*	*Accession #*	*Identity and Species*
1	AREFSLENTANIGIXA	RZC00518 (E=0.003)[d]	Protein translation elongation factor G	NP_654054 (E=0.15)	GTP_EFTU, Elongation factor Tu GTP binding domain	T44380 (E=0.15)	translation elongation factor EF-G fus *Bacillus halodurans*
2	SKIIGIDLGTTNS	RZC05735 (E=0.028)	Hypothetical protein	NP_658346 (E=6e-04)	HSP70, Hsp70 protein	P95693 (E=6e-04)	chaperone protein dnaK (Heat shock protein 70) *Streptococcus agalactiae*
3	SEQNKAKALPDRFEIEE	RZC04582 (E=3e-04)	Group B oligopeptidase pepB	NP_655102 (E=0.011)	Peptidase_M3, Peptidase family M3	NP_077141 (E=5.1)	RIKEN cDNA 0610009O20 *Mus musculus*
4	MKGKLIXXGGG	RZC05749 (E=2.0)	Succinate dehydrogenase flavoprotein subunit	NP_658544 (E=41)	FAD_binding_2, FAD binding domain	CAC80672 (E=432)	hypothetical protein *Listeria seeligeri*
5	AKDIKFSEEARRSMLRG	RZC01491 (E=1e-05)	60 kDa chaperonin	NP_654198 (E=2e-08)	cpn60_TCP1, TCP-1/ cpn60 chaperonin family	O50305 (E=1e-07)	class I heat-shock protein *Bacillus halodurans*
6a	MVVAYKHEPFTDFSVEAN	RZC04627 (E=2e-07)	1-pyrroline-5-carboxylate dehydrogenase	NP_654240 (E=3e-10)	aldedh, Aldehyde dehydrogenase family	NP_244808 (E=9e-06)	1-pyrroline-5-carboxylate dehydrogenase *Bacillus halodurans*
6b	MSQLAVNXXEKVEKYLQP	RZC01594 (E=0.004)	Aldehyde dehydrogenase	NP_657461 (E=2e-04)	aldedh, Aldehyde dehydrogenase family	CAC28725 (E=9.2)	hypothetical protein *Neurospora crassa*
6c	MAFEFKLPDIGEGIHESE	RZC00835 (E=2e-07)	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase	NP_658008 (E=4e-09)	2-oxoacid_dh, 2-oxo acid dehydrogenase acyltransferase (catalytic domain)	P11961 (E=4e-09)	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (E2) *Bacillus stearothermophilus*
7a	SIRAEEISALIKQQIEN	RZC00099 (E=2e-04)	ATP synthase alpha chain	NP_653764 (E=1e-09)	ATP-synt_ab, ATP synthase alpha/beta family, nucleotide-binding domain	P09219 (E=1e-09)	ATPA_BACP3 ATP synthase alpha chain *Thermophilic bacterium*
7b	XVGDFPEXL	RZC00979 (E=0.12)	Dihydrolipoamide dehydrogenase	NP_658007 (E=1.2)	pyr_redox, Pyridine nucleotide-disulfide oxidoreductase	P21880 (E=9.4)	Dihydrolipoamide dehydrogenase E3 subunit of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes *Bacillus subtilis*
8	MNKGRVTQIMGPVVDXK	RZC03299 (E=5.4)	ABC transporter ATP binding protein	NP_653762 (E=2e-07)	ATP-synt_ab, ATP synthase alpha/beta family, nucleotide-binding domain	P12698 (E=4e-06)	ATPB_BACME ATP synthase beta chain *Bacillus megaterium*
9	MSTIIDVYAREVLDSRGN	RZC02871 (E=2e-06)	Enolase	NP_653583 (E=1e-09)	enolase, Enol-ase	NP_623349 (E=1e-07)	Enolase *Thermoanaerobacter tengcongensis*
10	MEEAPFYXDTWVEVDLD	RZC04416 (E=1e-06)	Alanine racemase	NP_654182 (E=2e-08)	Ala_racemase, Alanine racemase	Q9KFF9 (E=0.001)	alanine racemase *Bacillus halodurans*

11	TLEIFEYLEKYDYEQVV	RZC02766 (E=2e-06)	Leucine dehydrogenase	NP_658202 (E=8e-09)	GLFV_dehydrog, E/ Leucine/ Phenyalanine/ Valine dehydrogenase	1LEH_A (E=1e-04)	A Chain A, Leucine Dehydrogenase *Bacillus sphaericus*
12a	MKTLLEVKDLQVSFDT	RZC03621 (E=6e-05)	Oligopeptide transport ATP-binding protein oppD	NP_655091 (E=1e-07)	ABC_tran, ABC transporter	AAG22037 (E=1e-07)	OppD *Bacillus thuringiensis*
12b	MTKIGINGFGRIGXNVFR	RZC05947 (E=2e-04)	GapDH	NP_653687 (E=1e-06)	gpdh_C, Glyceraldehyde 3-phosphate dehydrogenase, C-terminal domain	NP_44427 (E=2e-05)	Glyceraldehyde 3-phosphate dehydrogenase *Bacillus halodurans*
13	AQMTMIQAITDALRVEM	RZC01921 (E1e-05)	Pyruvate dehydrogenase E1 component of beta subunit	NP_658009 (E=7e-05)	transket_pyr, Transketolase, pyridine binding domain	NP_243520 (E=7e-05)	pyruvate dehydrogenase E1 (lipoamide) beta subunit *Bacillus halodurans*
14	VISMKQLLEAGVXFG	RZC00298 (E=6e-05)	Ribosomal protein S2P	NP_657796 (E=1e-08)	Ribosomal_S2, Ribosomal protein S2	NP_346623 (E=1e-04)	Ribosomal protein S2 *Streptococcus pneumoniae* TIGR4
15	FFSDKEVSNNTFAAGTL	RZC01638 (E=4e-06)	Spore coat-associated protein N	NP_655177 (E=0.064)	hypothetical protein predicted by GeneMark	CAD30177 (E=55)	putative spore coat-associated protein *Bacillus thuringiensis serovar israelensis*
16	MAKHELPNLPYAYDALEP	RZC01689 (E=011)	Superoxide dismutase (Mn)	NP_658307 (E=1e-08)	sodfe, Iron/manganese superoxide dismutase, alpha-hairpin domain	AAL09677 (E=3e-06)	superoxide dismutase *Staphylococcus epidermidis*
17	MSRIGKKILEIPAGVTIT	RZC06098 (E=14)	Hypothetical protein	NP_654068 (E=4e-07)	Ribosomal_L6, Ribosomal protein L6	AAA22700 (E=0.014)	ribosomal protein L6 *Geobacillus stearothermophilus*
18a	MTKDFYTALKERRTY	RZC02874 (E=4e-06)	Hypothetical cytosolic protein	NP_657300 (E=2e-07)	Nitroreductase, Nitroreductase family	NP_349906 (E=0.49)	Nitroreductase family protein *Clostridium acetobutylicum*
18b	MKATGIVRRIDDLGRVV	RZC04270 (E=4e-06)	Stage V sportulation protein T	NP_654002 (E=2e-08)	hypothetical protein predicted by GeneMark	NP_349810 (E=2e-08)	Stage V sporulation protein T, AbrB family transcriptional regulator *Clostridium acetobutylicum*
19	MLLIGTEVKPFKANAYH	RZC03162 (E=8e-07)	Alkyl hydroperoxide reductase c22 protein	NP_654276 (E=6e-09)	AhpC-TSA, AhpC/TSA family	NP_641259 (E=0.001)	alkyl hydroperoxide reductase subunit C *Xanthomonas axonopodis* pv. citri str. 306
20	FFSDKEVSNNTFAAGTL	RZC01638 (E=4e-06)	Spore coat-associated protein N	NP_655177 (E=4e-07)	hypothetical protein predicted by GeneMark	CAD30177 (E=8e-04)	putative spore coat-associated protein *Bacillus thuringiensis serovar israelensis*

a. *B. cereus* gapped genome data (www.integratedgenomics.com)

b. *B. anthracis* genome data [9] and associated Genbank accession numbers

c. NCBI GenBank best match not including *B. cereus* or *B. anthracis*

d. expectation value

Table 2: *Molecular weights and pI of spots resolved by 2D PAGE*

SPOT #	MEASURED VALUE FROM 2D-SDS GEL		IDENTITY	THEORETICAL VALUE FROM GENOME DATA			
				B. cereus		*B. anthracis*	
	kDa	*pI*		*kDa*	*pI*	*kDa*	*pI*
1	72-77	5.3-5.8	translation elongation factor G	18.8[b]	5.78[b]	76.3	4.83
2	67-72	4.7-5.0	heat shock protein 70	11.0[b]	8.73[b]	65.8	4.55
3	59-64	5.2-5.6	oligopeptidase B	70.2	4.84	70.4	4.84
4	57-61	6.0-6.5	succinate dehydrogenase	23.7[b]	6.33[b]	65.9	5.84
5	53-60	4.9-5.2	60 kDa chaperonin	21.2[b]	4.79[b]	57.4	4.70
6a	49-54	5.9-6.4	pyrroline-5-carboxylate dehydrogenase	56.2	5.44	56.2	5.44
6b	49-54	5.9-6.4	NADP-dependant aldehyde dehydrogenase	53.7	5.52	53.7	5.31
6c	49-54	5.9-6.4	Dihydrolipoamide acetyltransferase (E2)	26.2[b]	4.89[b]	44.9	5.58
7a	45-49	5.6-6.1	ATP synthase alpha subunit	54.6	5.24	54.6	5.24
7b	45-49	5.6-6.2	dihydrolipoamide dehydrogenase (E3)	23.0[b]	5.52[b]	49.5	5.23
8	43-48	5.0-5.4	ATP synthase beta subunit	41.4[a&b]	4.81[a&b]	51.0	4.85
9	41-47	4.7-5.1	enolase	46.4	4.55	46.4	4.55
10	36-44	5.2-5.7	alanine racemase	43.8	5.35	43.7	5.56
11	37-41	5.6-6.0	leucine dehydrogenase	39.9	5.08	39.9	5.08
12a	33-38	5.9-6.3	oligopeptide ABC transporter	38.1	5.39	38.1	5.67
12b	33-38	5.9-6.3	glyceraldehyde 3-phosphate dehydrogenase	20.4[a]	5.42[a]	35.8	5.38
13	31-34	4.7-5.1	pyruvate dehydrogenase (E1) beta subunit	35.2	4.65	35.2	4.65
14	21-23	9.4-9.8	ribosomal protein S2	21.6[a]	7.24[a]	26.5	9.13
15	17-19	4.3-4.6	camelysin 1	19.1	4.36	19.1	4.28
16	17-18	5.6-6.0	superoxide dismutase (Mn)	21.6[a&b]	5.35[a&b]	22.7	5.37
17	16-17	9.4-9.9	ribosomal protein L6	c	c	19.5	9.42
18a	15-17	5.6-5.9	nitroreductase family protein	22.7	5.16	22.7	5.70
18b	15-17	5.6-5.9	stage V sporulation protein T	19.7	5.00	21.6	4.93
19	16-17	5.3-5.6	alkyl hydroperoxide reductase subunit C	20.7	4.69	20.7	4.72
20	14-15	5.3-5.6	camelysin 2	19.0	5.05	19.0	5.23

a. N-terminus not present in *B. cereus* genome data

b. C-terminus not present in *B. cereus* genome data

c. Entire sequence not present in *B. cereus* genome data

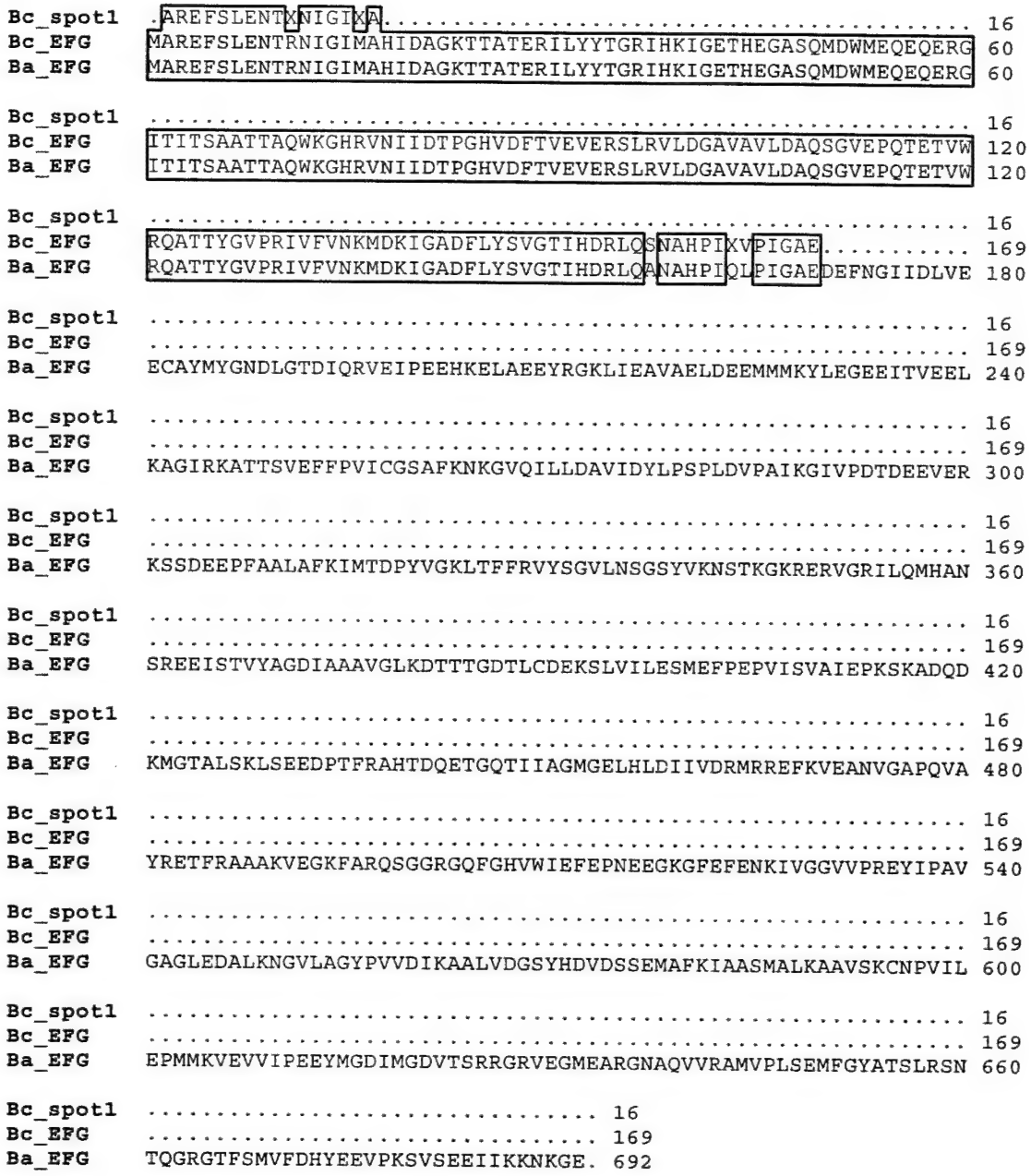

Figure 3. *Translation Elongation Factor G. Bc_spot 1 denotes the sequence derived from Edman degradation of spot 1 from a 2D gel. Bc_EFG is the amino acid sequence of translation elongation factor G found in the B. cereus database. The c-terminus of this protein falls within a gap in the database. Ba_EFG is the amino acid sequence of translation elongation factor G found in the B. anthracis database.*

In studies of *Escherichia coli*, hsp70 was found to be a ribosome-associated protein, implicated in assisting nascent polypeptides to acquire a three-dimensional structure on the ribosome [11]. In another study it was shown that the synthesis of dnaK protected *E. coli* from H_2O_2 killing under conditions when functional ethanol oxidoreductase was

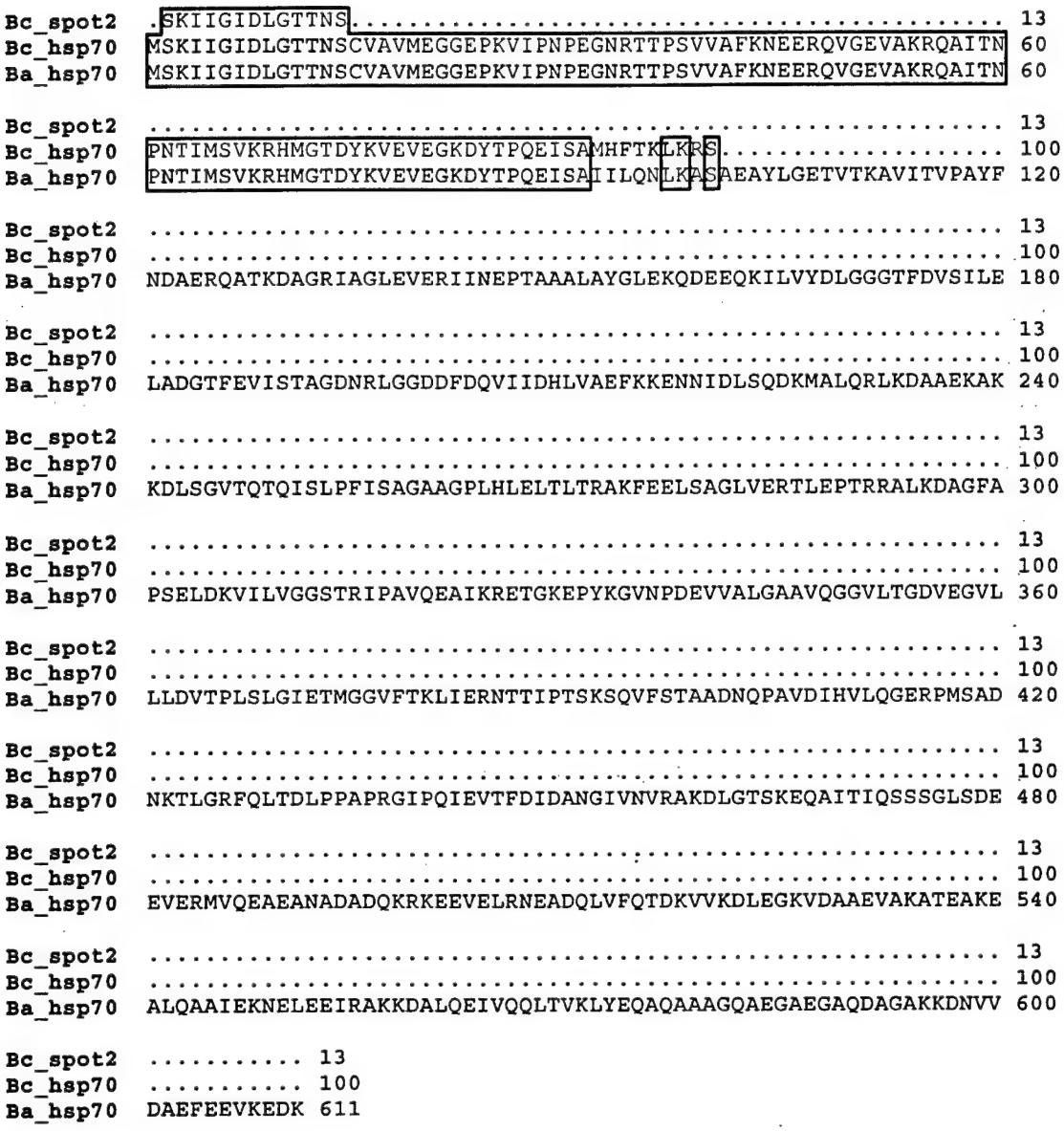

Figure 4. *Heat Shock Protein 70. Bc_spot 2 denotes the sequence derived from Edman degradation of spot 2 from a 2D gel. Bc_hsp70 is the amino acid sequence of heat shock protein 70 found in the B. cereus database. The c-terminus of this protein falls within a gap in the database. Ba_hsp70 is the amino acid sequence of heat shock protein 70 found in the B. anthracis database.*

lacking [12]. Therefore, in addition to the known role of protecting cells against heat stress, dnaK also protects numerous kinds of proteins from oxidative damage [12]. In light of this role, it is possible that dnaK would be found within the spore in order to help in the formation of new proteins during germination. As well, dnaK would be beneficial to the spore in protecting it against environmental factors such as heat and oxidative damage.

Spot 3 contained a sequence that was identified as oligopeptidase B (opdB). In *B. cereus* the sequence matched with group B oligopeptidase while in the *B. anthracis* database the sequence was identified as a protein within the peptidase family (Table 1 and Figure 5). The strongest hit, outside of *B. cereus* and *B. anthracis*, was a *Mus musculus* cDNA, although the match itself was poor (Table 1). Comparison of the ranges of molecular weight and pI of the excised spot showed that it was close but slightly smaller and less acidic that oligopeptidase in *B. cereus* and *B. anthracis* (Table 2). Oligopeptidase B is common in gram-negative bacteria where it has amidolytic activity against substrates with basic residues, exclusively hydrolyzing peptide bonds C-terminal to proline residues [13]. Since it is almost exclusively found in ancient unicellular eukaryotic organisms, Gram-negative bacteria and spirochetes, it has great potential for specific therapeutic applications [13,14]. It is interesting to note that opdB was found in *B. cereus,* which is a Gram-positive organism, and that the N-terminus had no homology to other bacterial opdB sequences.

The sequence derived from spot 4 matched with succinate dehydrogenase (sucDH), although only 11 amino acids could be resolved. In the *B. cereus* database the sequence corresponded with the succinate dehydrogenase flavoprotein subunit although the c-terminus of this protein fell in a gap of the genome (Table 1 and Figure 6). In the *B. anthracis* database the sequence was matched with an FAD binding domain protein (Table 1 and Figure 6). An NCBI BLAST search only found a hypothetical *Listeria seeligeri* protein with a very poor expect value of 432 (Table 1). Despite this, spot 4 was identified as sucDH due to its close molecular weight, pI, and alignment with the *B. anthracis* sequence, as seen in Table 2. SucDH is a flavoprotein (FAD) containing iron and is one of the critical enzymes of the citric acid or the Krebs cycle [15,16]. Within the citric acid cycle, which ultimately converts acetate into CO_2 and NADH, sucDH converts succinate and ubiquinone into fumarate and ubiquinol [17] [15]. As well succinate dehydrogenase is the only enzyme in the citric acid cycle that is membrane bound to the inner cytoplasmic membrane of the bacteria [15]. Since the citric acid cycle is one of the central metabolic pathways in prokaryote cells it is possible that the enzymes required for this cycle would be stored in the spore. The products of such pathways would be needed for the processing of other proteins as well as for energy during germination.

The amino acid sequence derived from spot 5 was identified as 60 kDa chaperonin (cpn60) based on near perfect matches with cpn60 of *B. cereus*, *B. anthracis*, and *B. halodurans* (Figure 7 and Table 1). Spot 5 had a molecular weight range that included the molecular weight of cpn60 from *B. anthracis* and its pI range was also similar (Table 2). The c-terminus of cpn60 in *B. cereus* fell within a gap of the genome therefore the accurate molecular weight and pI could not be calculated (Table 2). Cpn60 is also known as GroEL in bacteria. Together GroEL and GroES assist with the folding of newly synthesized proteins and the refolding of conformationally damaged proteins [18]. From this it is plausible to assume that this protein could be found within the spore to insure that other essential germination factors are correctly assembled. It has also been shown that in *Bacillus subtilis* spores, GroEL can be induced by heat shock [7]. This induction is important during times of stress since GroEL may help to repair protein damage in the spore. GroEL has also been reported as a surface associated protein in the exosporium of *B. cereus*, where it was hypothesized unlikely to have a protein-folding role [4]. Interestingly, cpn60 has also been found to display strong antigenic signals in many bacterial species and has the potential to induce immunity against unrelated

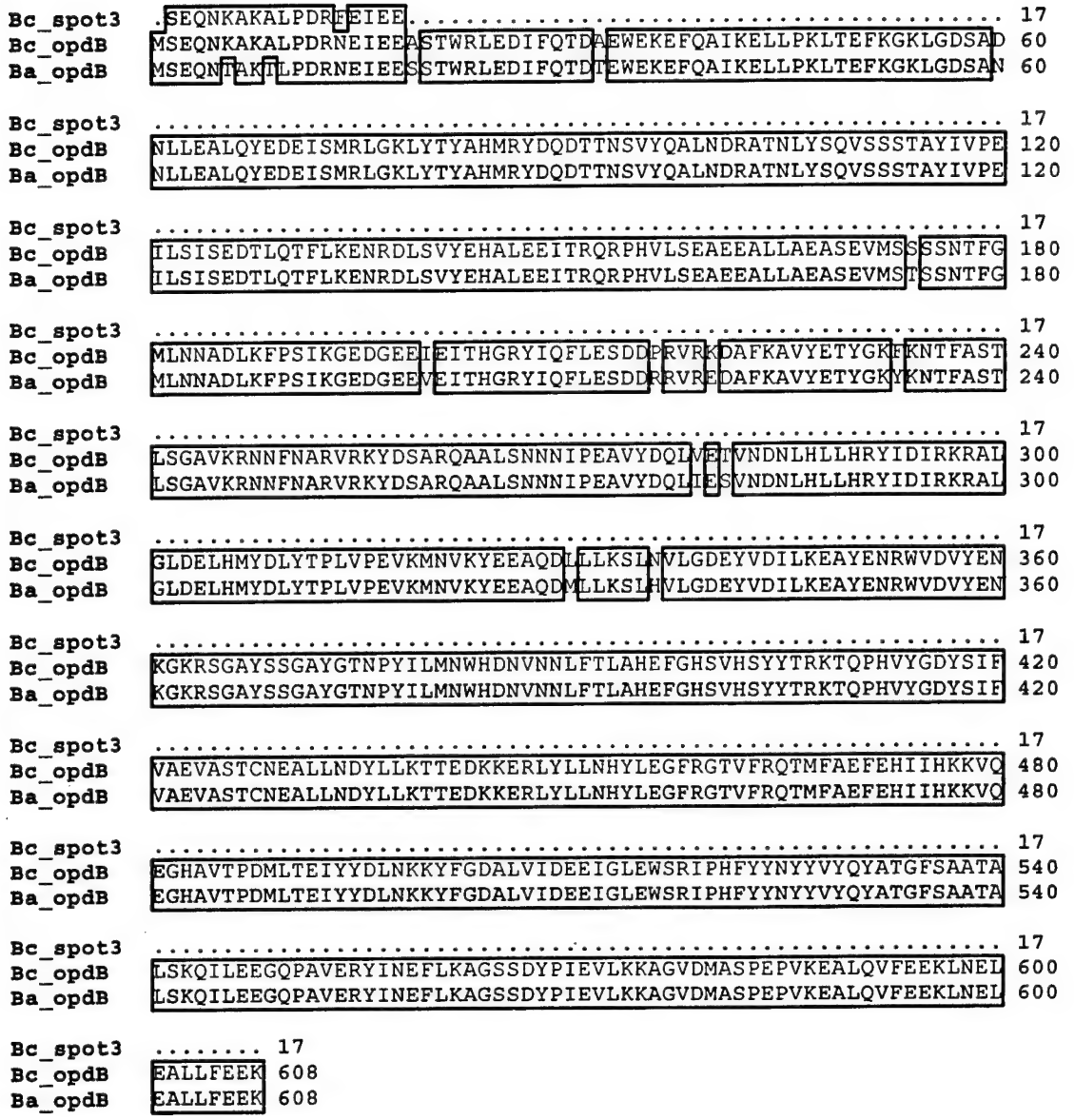

Figure 5. *Oligopeptidase B. Bc_spot 3 denotes the sequence derived from Edman degradation of spot 3 from a 2D gel. Bc_opdB is the amino acid sequence of oligopeptidase B found in the B. cereus database. Ba_opdB is the amino acid sequence of oligopeptidase B found in the B. anthracis database.*

bacterial infections [19]. This cross-protection is possible since a high degree of antigenic homology in cpn60 exists between bacteria of different species [19]. For example, it has been found that there is a very close alignment in the sequence of cpn60 in humans, *Chlamydia trachomatis*, *Escherichia coli*, and *Mycobacterium tuberculosis* [19]. The presence of GroEL in the spore exosporium may have some applicability for therapeutic or diagnostic consideration.

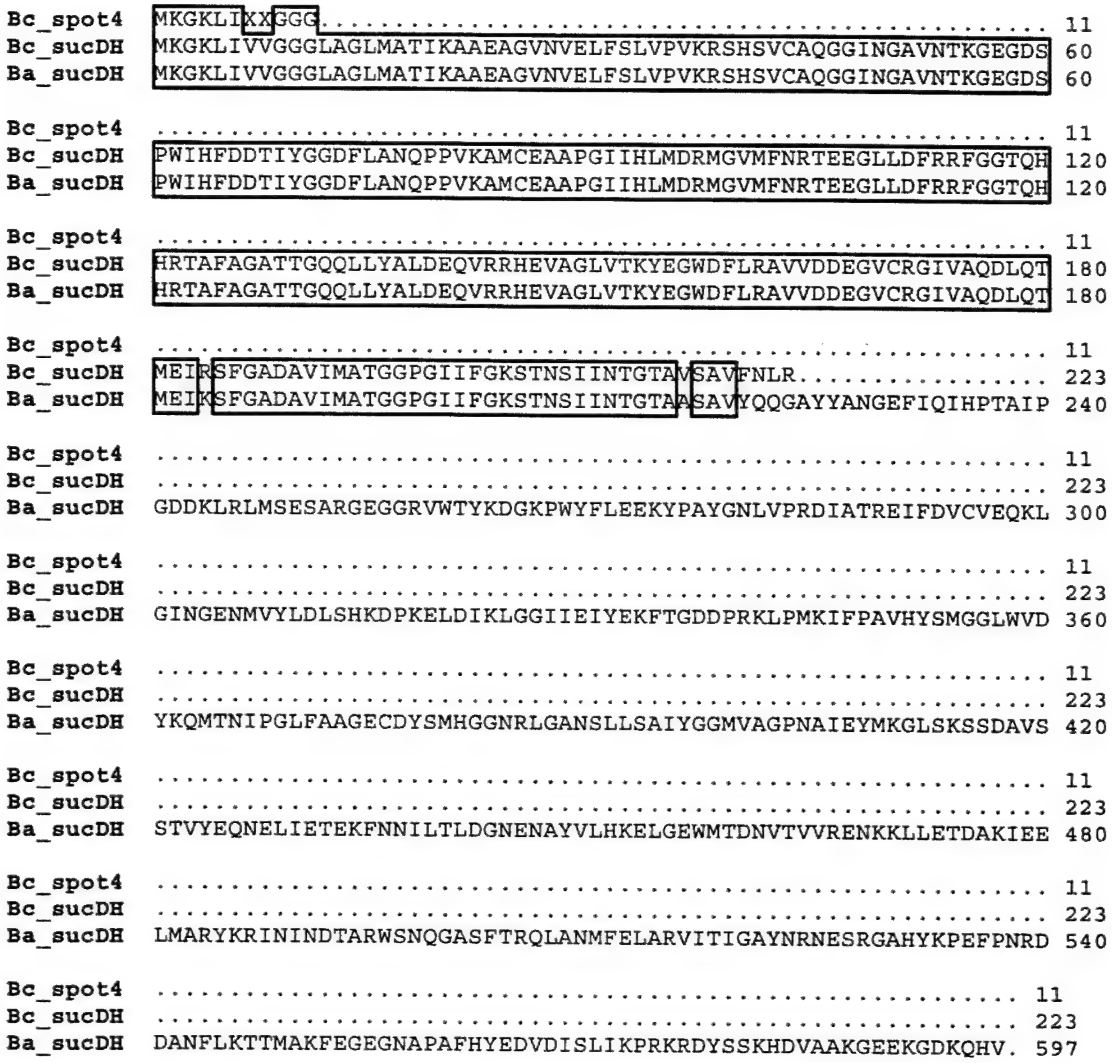

Figure 6. *Succinate Dehydrogenase. Bc_spot 4 denotes the sequence derived from Edman degradation of spot 4 from a 2D gel. Bc_sucDH is the amino acid sequence of succinate dehydrogenase found in the B. cereus database. The c-terminus of this protein falls within a gap in the database. Ba_sucDH is the amino acid sequence of succinate dehydrogenase found in the B. anthracis database.*

The amino acid sequence derived from protein spot 6a was determined to be the sequence of pyrroline-5-carboxylate dehydrogenase (p5cDH). The alignment of this sequence was an excellent match with p5cDH of *B. cereus* and *B. anthracis* as well as *B. halodurans* (Figure 8 and Table 1). The measured ranges of the molecular weight and pI of spot 6a were close to that of *B. cereus* and *B. anthracis* p5cDH (Table 2). P5cDH is associated with the cytoplasmic membrane [20] and is involved in the ornithine degradation pathway [4]. In this pathway it converts both proline and ornithine to glutamate [4]. It has been proposed that glutamate may provide carbon and nitrogen precursors for spore biogenesis and may provide energy for the cell during spore formation [4]. Although it has been documented in spores it is unclear as to whether or not p5cDH is functional in this state [4].

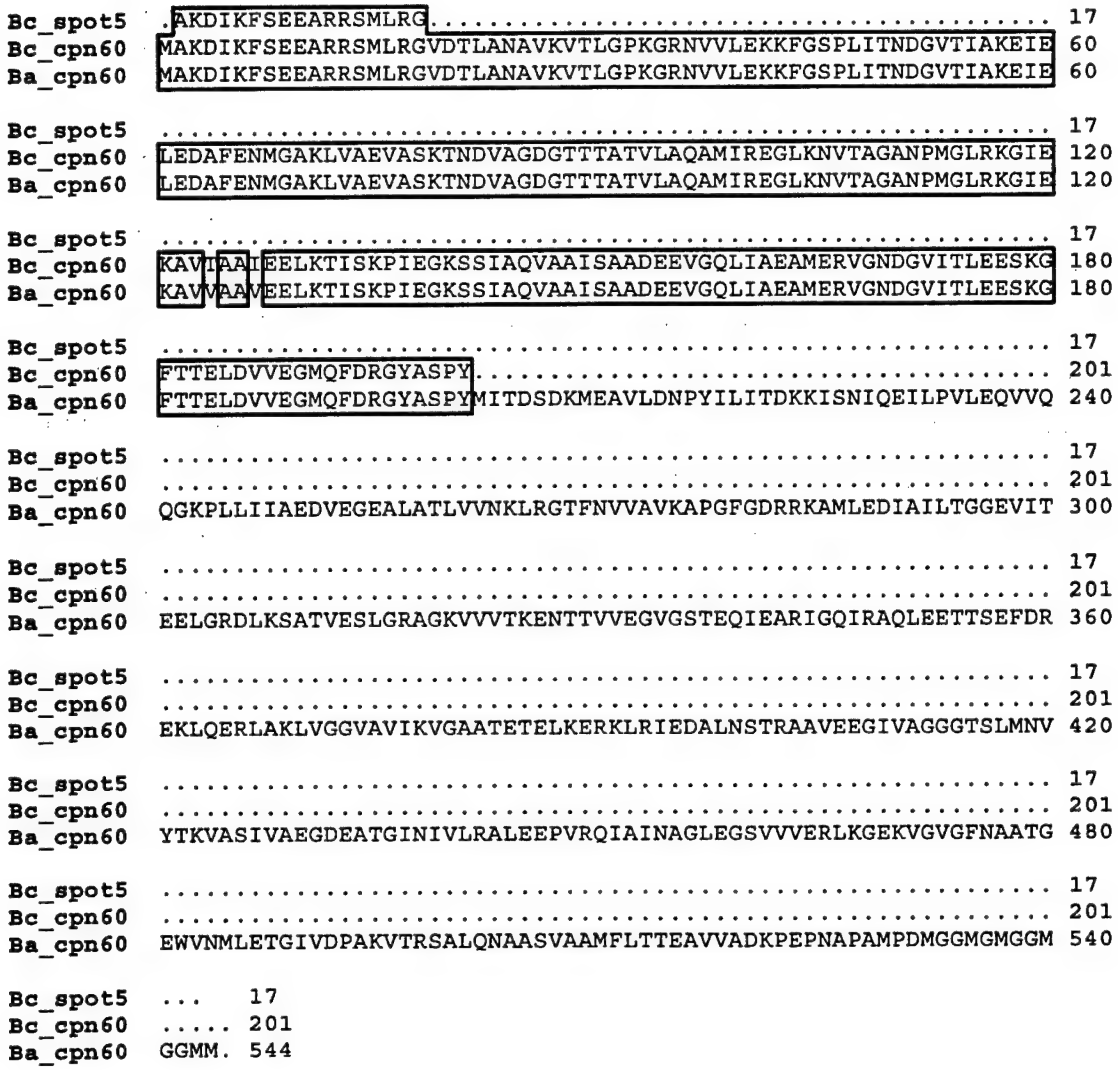

***Figure 7.** 60 kDa chaperonin. Bc_spot 5 denotes the sequence derived from Edman degradation of spot 5 from a 2D gel. Bc_cpn60 is the amino acid sequence of 60 kDa chaperonin found in the B. cereus database. The c-terminus of this protein falls within a gap in the database. Ba_cpn60 is the amino acid sequence of 60 kDa chaperonin found in the B. anthracis database.*

p5cDH and proline dehydrogenase are part of an enzyme pair that oxidizes proline to glutamic acid [21]. It also appears that p5cDH activity is elevated during times of salt stress and nitrogen starvation [22]. Such conditions are likely to be encountered by the spore.

The second protein identified from spot 6 (designated 6b) was NADP-dependant aldehyde dehydrogenase (ALDH). The best alignments for this sequence were ALDH in *B. cereus* and *B. anthracis* (Table 1 and Figure 9). The next best match was identified as a hypothetical protein in *Neurospora crassa* (Table 1). The range of molecular weights and pI for the spot were an excellent match with the molecular weights and pI of ALDH in both *B. cereus* and

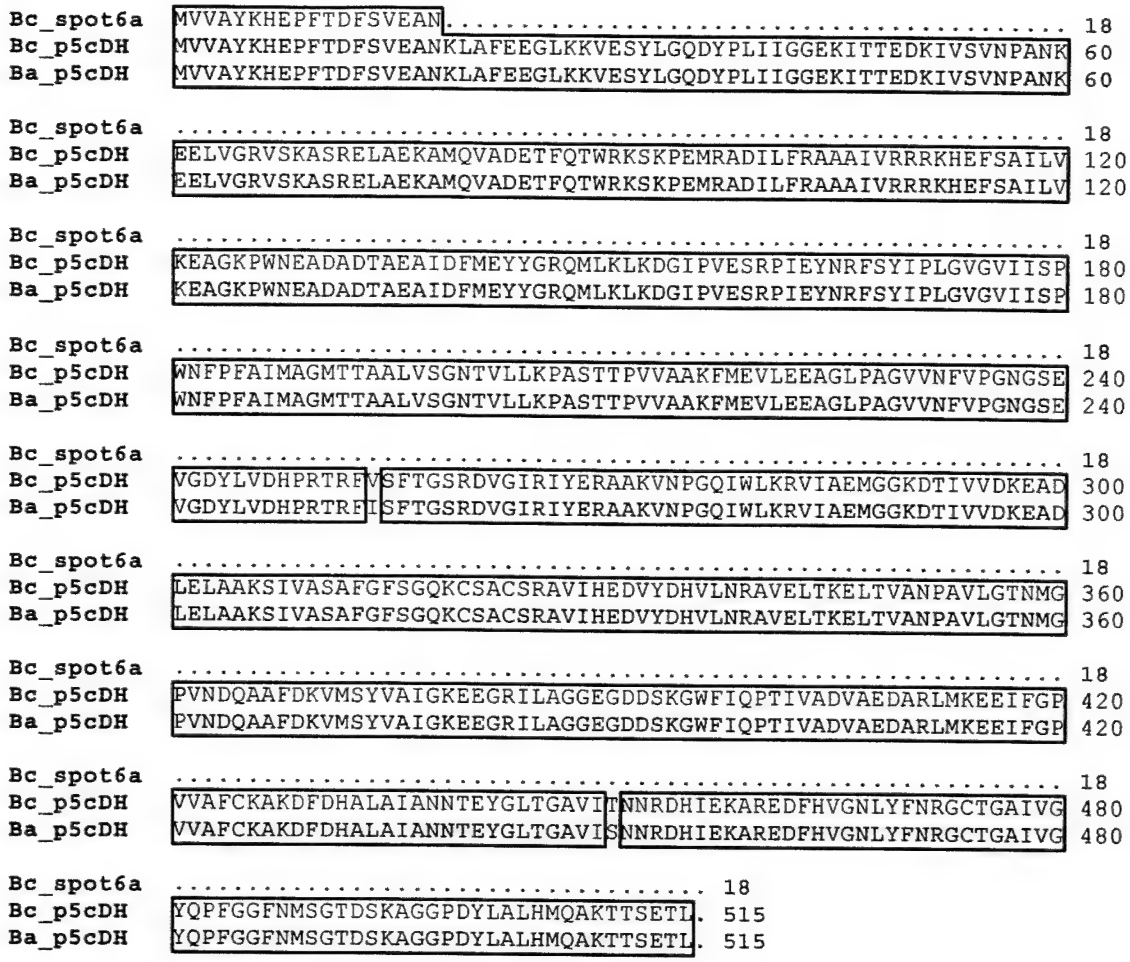

Figure 8. *1-pyrroline-5-carboxylate dehydrogenase. Bc_spot 6a denotes the sequence derived from Edman degradation of spot 6a from a 2D gel. Bc_p5cDH is the amino acid sequence of 1-pyrroline-5-carboxylate dehydrogenase found in the B. cereus database. Ba_p5cDH is the amino acid sequence of 1-pyrroline-5-carboxylate dehydrogenase found in the B. anthracis database.*

B. anthracis (Table 2). Aldehyde dehydrogenase is part of the metabolic pathway that oxidizes alcohol [23], and converts Aldehyde, $NADP^+$, and H_2O into acid and NADPH [23]. The function of this enzyme in the spore is unknown, but may be involved in detoxification of metabolic aldehydes produced during germination.

The third sequence derived from spot 6 (designated 6c) was identified as dihydrolipoamide acetyltransferase (E2) of the pyruvate dehydrogenase multienzyme complex. This sequence was found to be an excellent match for E2 in *B. cereus*, *B. anthracis* and *Bacillus stearothermophilus* (Table 1 and Figure 10). This identification was considered valid based on the comparison of the range of molecular weight and pI values of the excised spot with the calculated values for E2 in *B. anthracis* (Table 2). The molecular weight and pI of E2 in *B. cereus* could not be calculated because the c-terminus of the protein fell in a gap in the database. Dihydrolipoamide acetyltransferase is the second enzyme in the pyruvate dehydrogenase multienzyme complex (PDC) which links glycolysis with the citric acid cycle

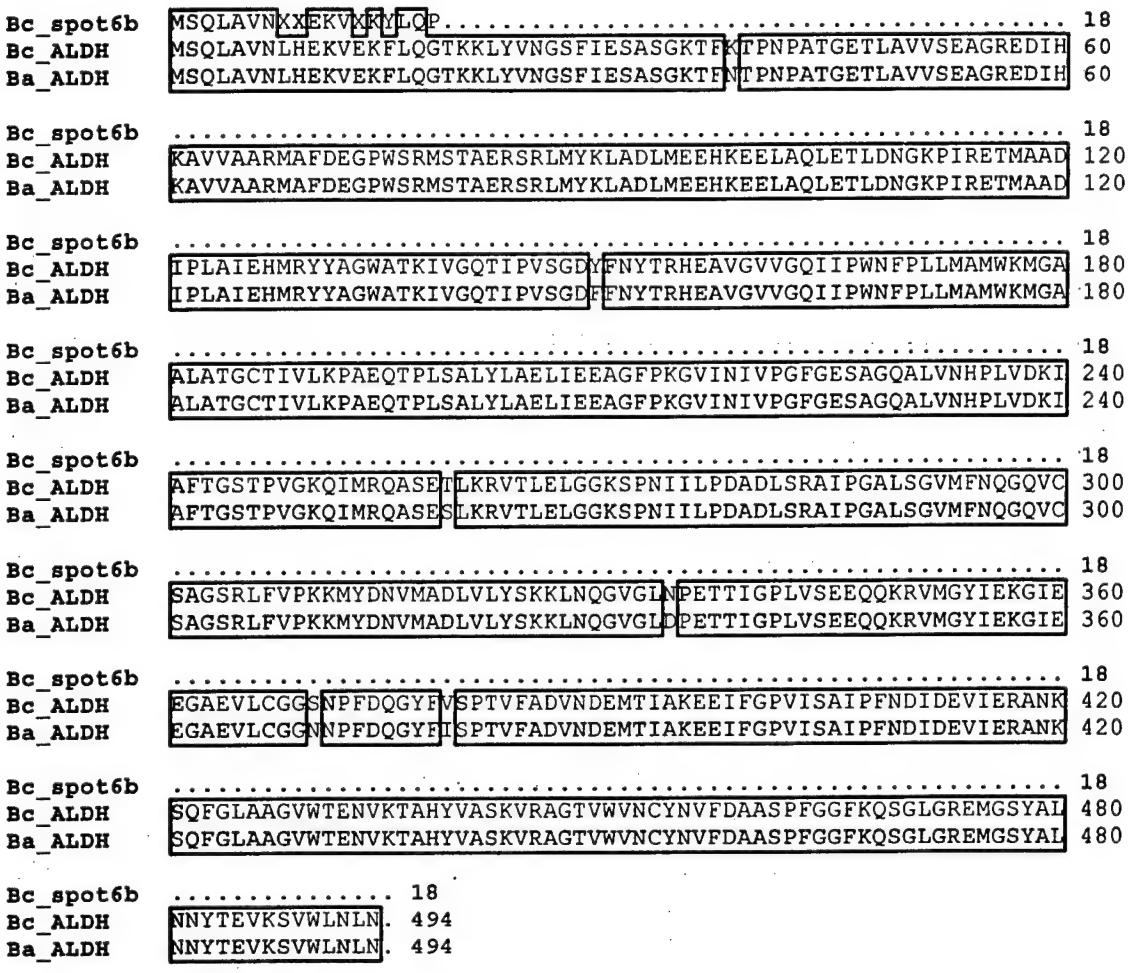

Figure 9. *NADP-dependant aldehyde dehydrogenase. Bc_spot 6b denotes the sequence derived from Edman degradation of spot 6b from a 2D gel. Bc_ALDH is the amino acid sequence of NADP-dependant aldehyde dehydrogenase found in the B. cereus database. Ba_ALDH is the amino acid sequence of NADP-dependant aldehyde dehydrogenase found in the B. anthracis database.*

[24]. PDC consists of pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3) [25] . The pyruvate dehydrogenase multienzyme complex is built around an octahedral core of dihydrolipoamide acetyltransferase (E2) chains, to which multiple copies of pyruvate dehydrogenase (E1) and dihydrolipoamide dehydrogenase (E3) bind tightly but non-covalently [26]. Sporulation in *B. subtilis* was shown to decrease in cells with mutant genes for E2 [25]. E2 subunits, which are present in the soluble fraction of sporulating cells, appear to function as checkpoints for stage II-III of sporulation [25]. Therefore it is likely that E2 would be contained within the spore as part of the main metabolic pathway in order to produce the products needed for germination. It is also interesting to note that it is specifically involved in monitoring sporulation and hence it is not surprising that it would found within the spore. As E2 is involved in monitoring sporulation, inhibition of this enzyme should prevent sporulation.

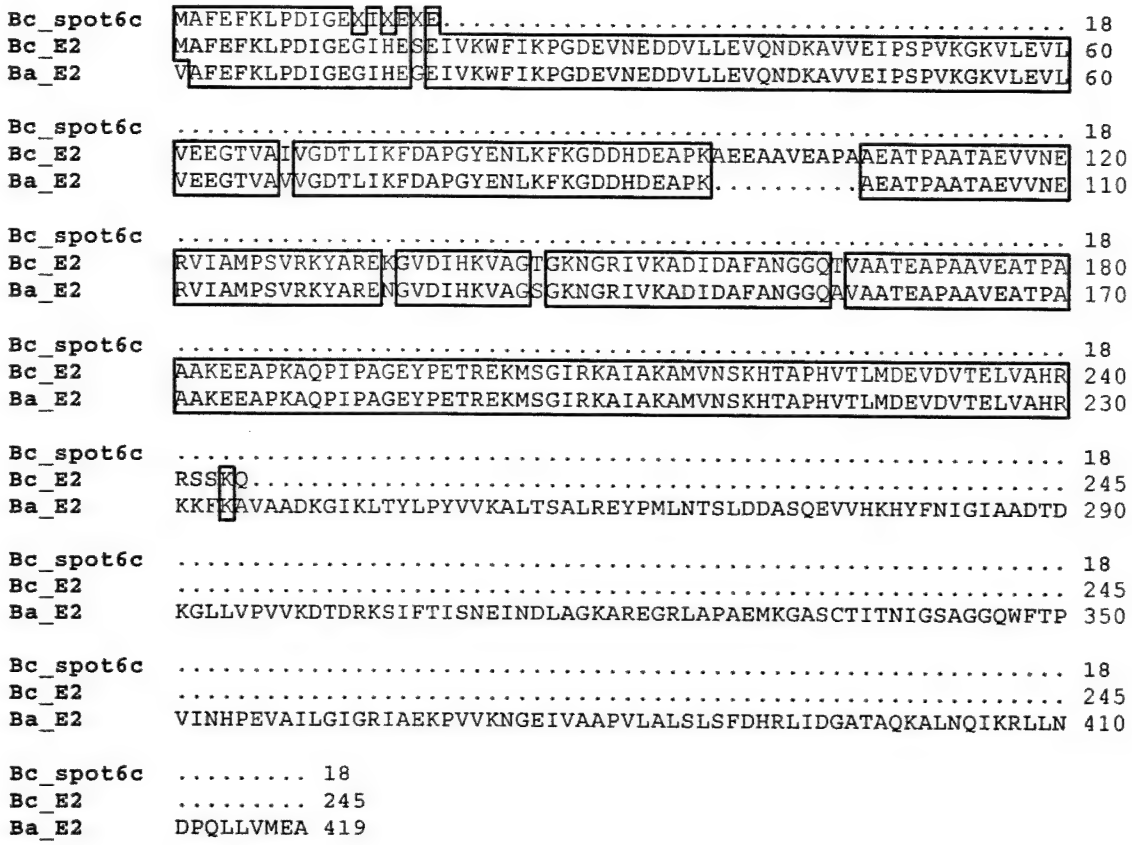

Figure 10. *Dihydrolipoamide acetyltransferase (E2). Bc_spot 6c denotes the sequence derived from Edman degradation of spot 6c from a 2D gel. Bc_E2 is the amino acid sequence of dihydrolipoamide acetyltransferase found in the B. cereus database. The c-terminus of this protein falls within a gap in the database. Ba_E2 is the amino acid sequence of dihydrolipoamide acetyltransferase found in the B. anthracis database.*

Spot 7a yielded an amino acid sequence that was a near perfect match for the alpha subunit of ATP synthase (ATPsynt-a) in *B. cereus*, *B. anthracis*, and *Thermophilic bacterium* (Table 1 and Figure 11). The excised spot fell within a molecular weight range of 45 to 49 kDa and a pI range of 5.6 to 6.1 which is only slightly smaller and basic than that of *B. cereus* and *B. anthracis* ATPsynt-a (Table 2). ATP synthase is made up of two subunits; F_o, which is embedded in the cytoplasmic membrane and F_1, which projects inward [15]. The catalytic part of the F_1 domain is made up of three beta and three alpha subunits surrounding a helical domain; ATPsynt-a is one of these alpha subunits [27]. The rotation of the alpha and beta subunits around this helical domain, driven by a flux of protons across the membrane down a proton gradient generated by electron transfer, changes the catalytic state of the F_1 domain [27]. This conformational change releases energy that is used in the dehydration of bound ADP and phosphate to ATP. In this manner, the enzyme is an essential part of membrane bioenergetics [28]. In some bacteria the main function of ATP synthase is to operate in the reverse direction using ATP generated by fermentative metabolism to provide a proton gradient to drive substrate accumulation and maintain ionic balance [15]. Due to its critical function in bioenergetics targeting and disabling this protein may help in the decontamination of *B. anthracis* or *B. cereus* spores.

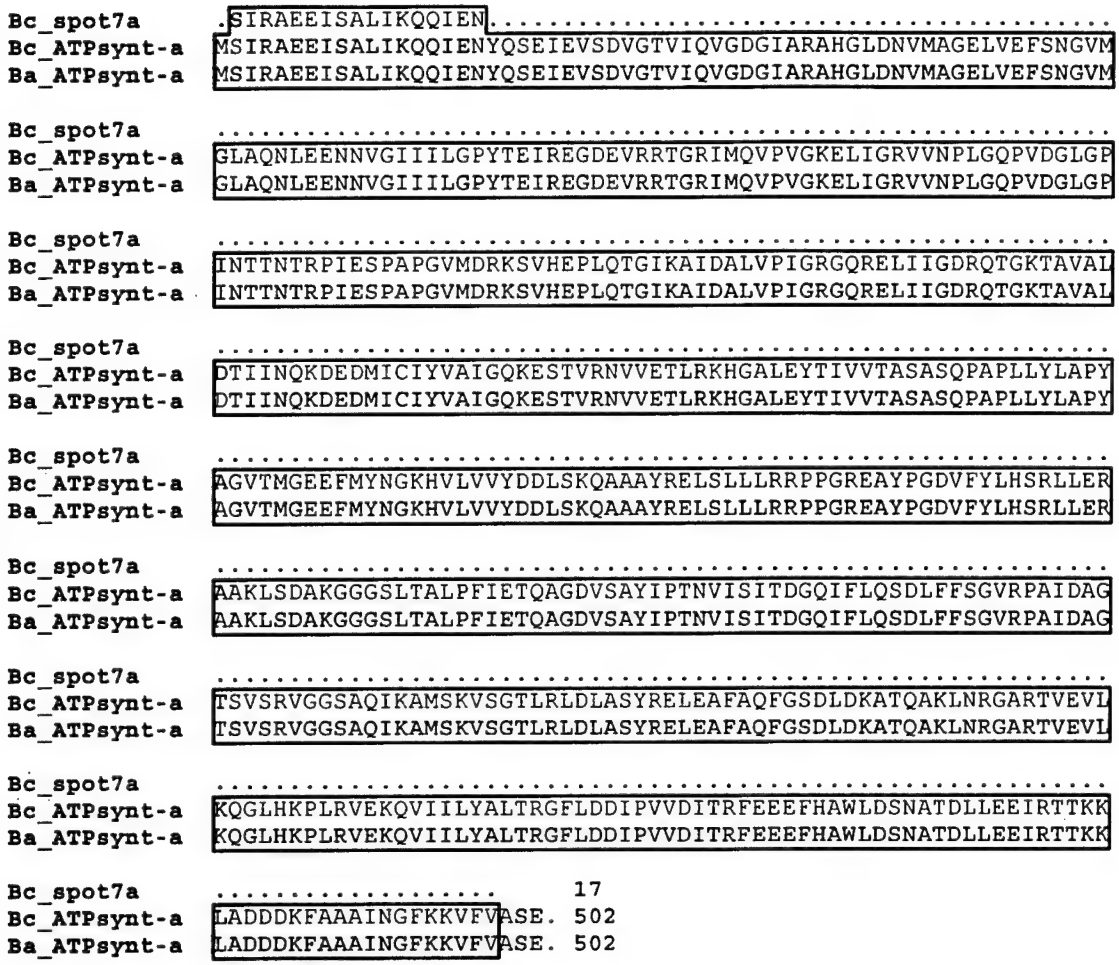

Figure 11. *ATP synthase alpha subunit. Bc_spot 7a denotes the sequence derived from Edman degradation of spot 7a from a 2D gel. Bc_ATPsynt-a is the amino acid sequence of the alpha subunit of ATP synthase found in the B. cereus database. Ba_ATPsynt-a is the amino acid sequence of the alpha subunit of ATP synthase found in the B. anthracis database.*

The second sequence derived from spot 7 (designated 7b) was identified as dihydrolipoamide dehydrogenase (E3) a component of the pyruvate dehydrogenase multienzyme complex. This sequence matched with E3 in *B. cereus* and *B. subtilis* (Table 1 and Figure 12). In the *B. anthracis* database the sequence matched with pyridine nucleotide-disulfide oxidoreductase (Table 1 Figure 12). The ranges of molecular weight and pI for the excised spot were good although slightly less acidic than the calculated data for E3 in *B. anthracis* (Table 2). E3 is part of the pyruvate dehydrogenase multienzyme complex described above [29], and is also part of the 2-oxoglutarate dehydrogenase complex [30]. The 2-oxoglutarate dehydrogenase complex consists of three different subenzymes, the E1o (2-oxoglutarate dehydrogenase) component, the E2o (dihydrolipoyl transsuccinylase) component, and the E3 (dihydrolipoamide dehydrogenase) component [31]. The exact function of E3 is unknown except that it acts within these two related multienzyme systems [32]. In one study it was shown that E3 was induced in B. *subtilis* under anaerobic conditions [33]. Upon heat treatment of the pyruvate dehydrogenase complex from *Bacillus stearothermophilus*, E3 is the most thermostable component [34]. Such stability is relevent given that that bacterial spores are often subjected to extreme temperatures.

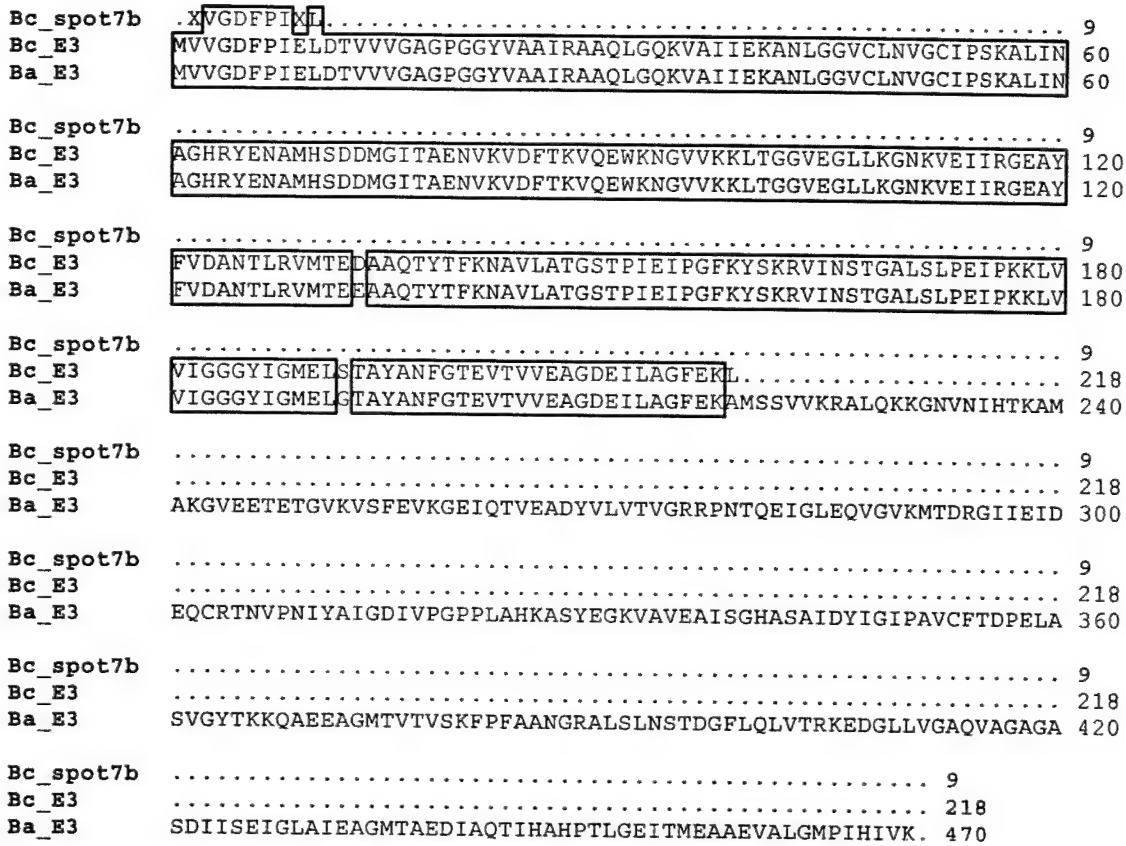

Figure 12. *Dihydrolipoamide dehydrogenase (E3). Bc_spot 7b denotes the sequence derived from Edman degradation of spot 7b from a 2D gel. Bc_E3 is the amino acid sequence of dihydrolipoamide dehydrogenase found in the B. cereus database. The c-terminus of this protein falls within a gap in the database. Ba_E3 is the amino acid sequence of dihydrolipoamide dehydrogenase found in the B. anthracis database.*

The amino acid sequence derived from spot 8 was identified as the beta subunit of ATP synthase (ATPsynt-b). This sequence matched with ATPsynt-b of *B. anthracis*, and *Bacillus megaterium* (Table 1 and Figure 13). The corresponding *B. cereus* sequence was missing the N-terminus due to a gap in the genome data. The range of molecular weight for this spot was slightly lighter while the range of pI was slightly less acidic than the calculated values for *B. anthracis* ATPsynt-b (Table 2). As stated above, ATP synthase is made up of two subunits; F_o, which is embedded in the cytoplasmic membrane and F_1, which projects inward [15]. As part of this complex there are three beta subunits within the F_1 domain. The details and implications of this system are described in detail in the paragraph on ATP synthase alpha subunit.

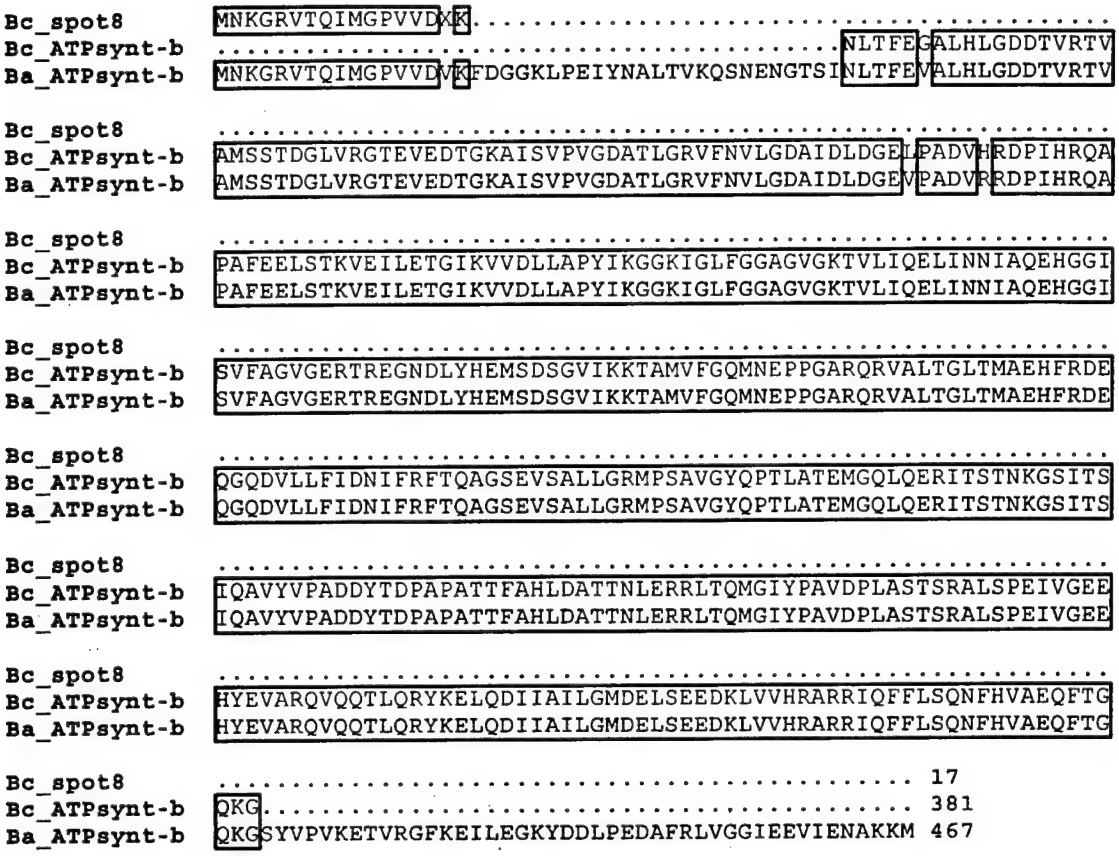

Figure 13. *ATP synthase beta subunit. Bc_spot 8 denotes the sequence derived from Edman degradation of spot 8 from a 2D gel. Bc_ATPsynt-b is the amino acid sequence of ATP synthase beta subunit found in the B. cereus database. The n-terminus and c-terminus of this protein fall in gaps in the database. Ba_ATPsynt-b is the amino acid sequence of ATP synthase beta subunit found in the B. anthracis database.*

The amino acid sequence derived from spot 9 was identified as enolase. There was a good match for this sequence with enolase of *B. cereus*, *B. anthracis*, and *Thermoanaerobacter tengcongensis* (Table 1 and Figure 14). The ranges for molecular weight and pI of the excised spot also matched well with the calculated values of enolase in both *B. cereus* and *B. anthracis* (Table 2). As part of the main glycolytic pathway, enolase catalyzes the step interconverting 2-phosphoglycerate (2-PGA) and phosphoenolpyruvate (PEP) by removing water [28]. The reaction is characterized by a proton shift from the enzyme to the substrate [15]. Enolase also catalyzes the reverse reaction during gluconeogenesis [15]. Since ATP is need for germination of the spore, and the main source of ATP is glycolysis, it is reasonable to assume that enolase would be contained within the spore.

The amino acid sequence derived from spot 10 was identified as alanine racemase. This sequence matched with alanine racemase of *B. cereus*, *B. anthracis* and *B. halodurans* (Table 1 and Figure 15). The ranges of molecular weight and pI for the excised spot were within the calculated values for alanine racemase in both *B. cereus* and *B. anthracis* (Table 2). Alanine racemase is an enzyme that converts L-alanine to D-alanine thereby preventing the germination of spores [1]. After germination, alanine racemase provided

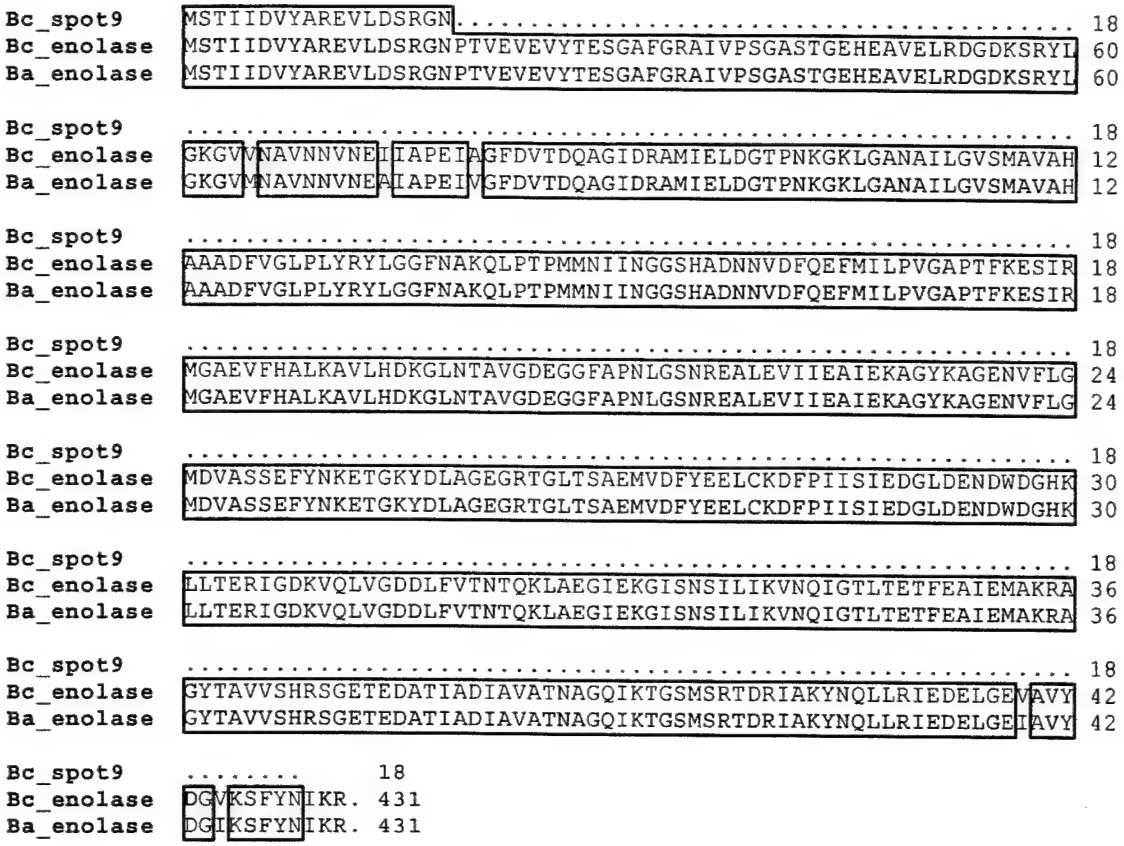

Figure 14. *Enolase. Bc_spot 9 denotes the sequence derived from Edman degradation of spot 9 from a 2D gel. Bc_enolase is the amino acid sequence of enolase found in the B. cereus database. Ba_enolase is the amino acid sequence of enolase found in the B. anthracis database.*

D-alanine which is required for cell wall biosynthesis [1]. For these two reasons alanine racemase is an important enzyme contained with in the *B. cereus* spore. The presence of alanine racemase controls the germination of high concentrations of spores if there is insufficient nutrients available. It is also important to note that O-carbamyl-D-serine has been found to inhibit alanine racemase [1]. Inhibition of alanine racemase would cause uncontrolled germination of spores, possiblly under conditions which would be unfavorable for vegetative growth. However since alanine racemase also provides the D-alanine for cell wall biosynthesis germination of the spores would most likely be stunted for lack of cell wall production. This may have possibilities as an anti-BW agent in preventing the germination of anthrax spores or interfering with the proper growth of the bacterial cell wall.

The amino acid sequence derived from spot 11 was identified as leucine dehydrogenase (leucDH). The sequence matched well with leucDH of *B. cereus*, *B. anthracis*, and *Bacillus sphaericus* (Table 1 and Figure 16). These results were confirmed by the calculation of molecular weight and pI of leucDH in *B. cereus* and *B. anthracis* which matched with the range of molecular weight and pI of the excised spot (Table 2). Leucine dehydrogenase functions catabolically in the bacterial metabolism of branched-chain L-amino acids by

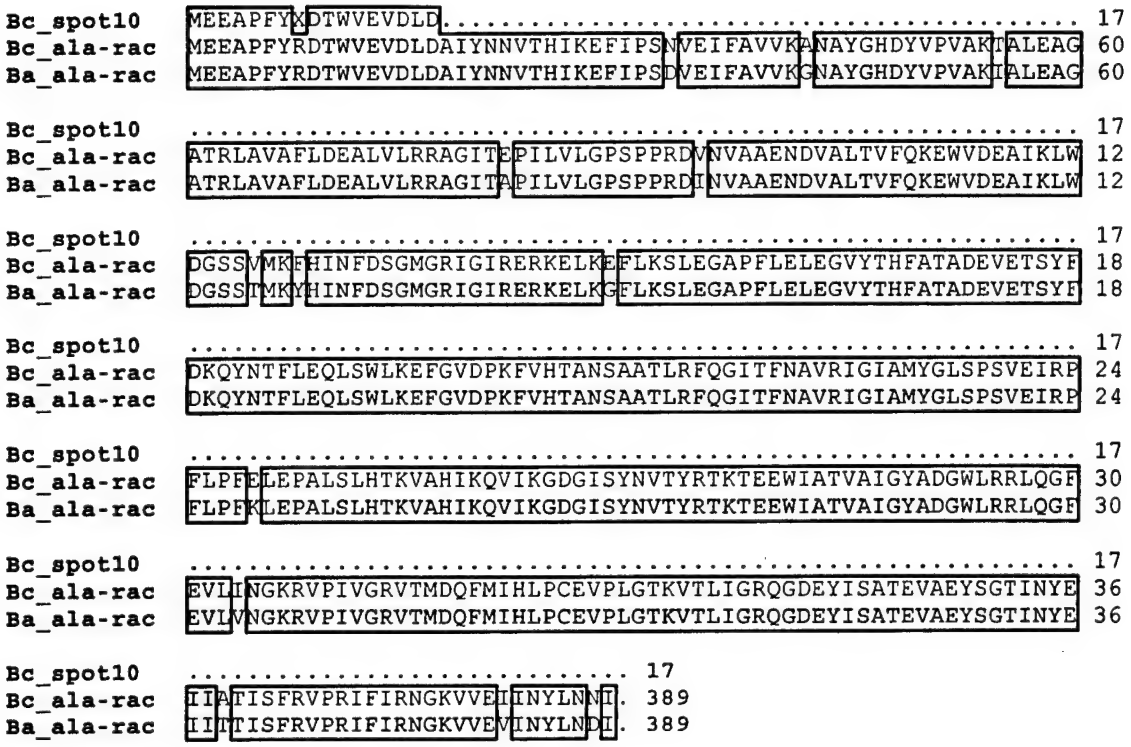

Figure 15. *Alanine Racemase. Bc_spot 10 denotes the sequence derived from Edman degradation of spot 10 from a 2D gel. Bc_ala-rac is the amino acid sequence of alanine racemase found in the B. cereus database. Ba_ala-rac is the amino acid sequence of alanine racemase found in the B. anthracis database.*

converting L-leucine, NAD^+ and H_2O into α-ketoisocaproate, NH_3, NADH, and H^+ [35]. The enzyme has been demonstrated to play a role in the germination of *B. subtilis* spores, presumably as a source of nitrogen for dipicolinic acid synthesis [36]. Therefore leucine dehydrogenase is likely to be found within a spore due to its role in germination.

The amino acid sequence derived from spot 12a was identified as oligopeptide ABC transporter (oppD). The sequence matched well with oppD of *B. cereus*, *B. anthracis*, and *B. thuringiensis* (Table 1 and Figure 17). The ranges of molecular weight and pI for the excised spot were accurate for the calculated molecular weight and pI of oppD in *B. cereus* and *B. anthracis* (Table 2). Oligopeptide transporters bind ATP and transport small oligopeptides into the cell from the outside environment [37]. Oligopeptide transport systems are essential for regulatory functions, as well as serving to nonspecifically transport peptides for anabolic and catabolic purposes [38]. OppD has also been reported to be an important sporulation initiator [38]. It has been shown that mutations in the *opp* oligopeptide transport system result in decreased sporulation, suggesting that a peptide molecule of some kind must be transported from outside the cell to complete the initiation of sporulation [38]. For this reason it is unsurprising that oppD was found within *B. cereus* spores.

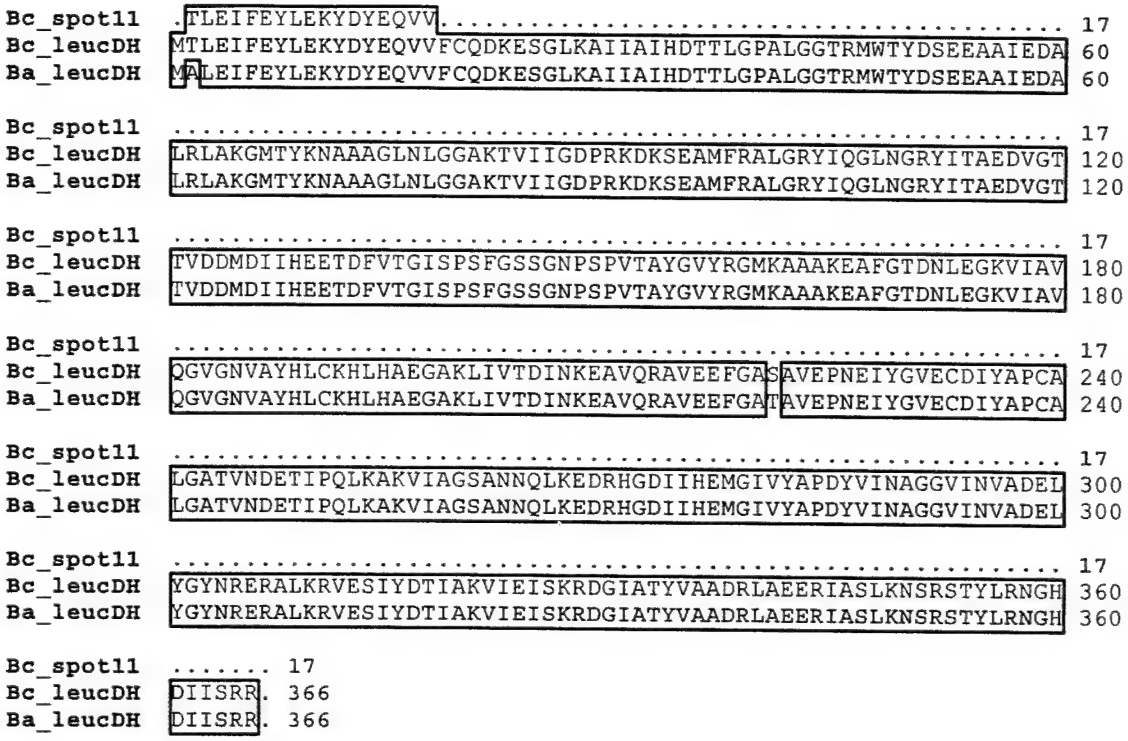

Figure 16. *Leucine Dehydrogenase. Bc_spot 11 denotes the sequence derived from Edman degradation of spot 11 from a 2D gel. Bc_leucDH is the amino acid sequence of leucine dehydrogenase found in the B. cereus database. Ba_leucDH is the amino acid sequence of leucine dehydrogenase found in the B. anthracis database.*

The second amino acid sequence derived from spot 12 (designated 12b) was identified as glyceraldehyde 3-phosphate dehydrogenase (gapDH). The sequence was an excellent match with gapDH of *B. anthracis*, and *B. halodurans* (Table 1 and Figure 18). The N-terminus of the corresponding *B. cereus* sequence was not present in the genome data due to the presence of a gap. The calculated molecular weight and pI of *B. anthracis* gapDH were within the ranges of molecular weight and pI for the excised spot. Glycolysis is one of the main pathways of carbon catabolism [39], converting glucose to pyruvate and then to fermentation products such as lactate and ethanol [27]. GapDH is a key enzyme in glycolysis and is induced by glucose and other sugars, and converts glyceraldehyde-3-phosphate into 1,3-biphosphoglyceric acid [27,39]. This reaction occurs twice for each molecule of glucose and is the first step of the oxidation reaction in glycolysis [27]. Due to its key position in this main pathway gapDH is highly conserved between species. The enzyme is likely to be found within the spore as part of this pathway which will be important during germination in the production of ATP and other metabolites for cell growth.

The amino acid sequence derived from spot 13 was identified as the beta subunit of pyruvate dehydrogenase (E1) within the pyruvate dehydrogenase multienzyme complex. The sequence matched with E1 beta subunit of *B. cereus* and *B. halodurans* (Table 1 and Figure 19). The sequenced also matched with a pyridine binding domain protein in *B. anthracis* (Table 1 and Figure 19). The ranges of molecular weight and pI for the excised spot contained the molecular weight of E1 found in *B. cereus* and *B. anthracis* (Table 2). The pyruvate

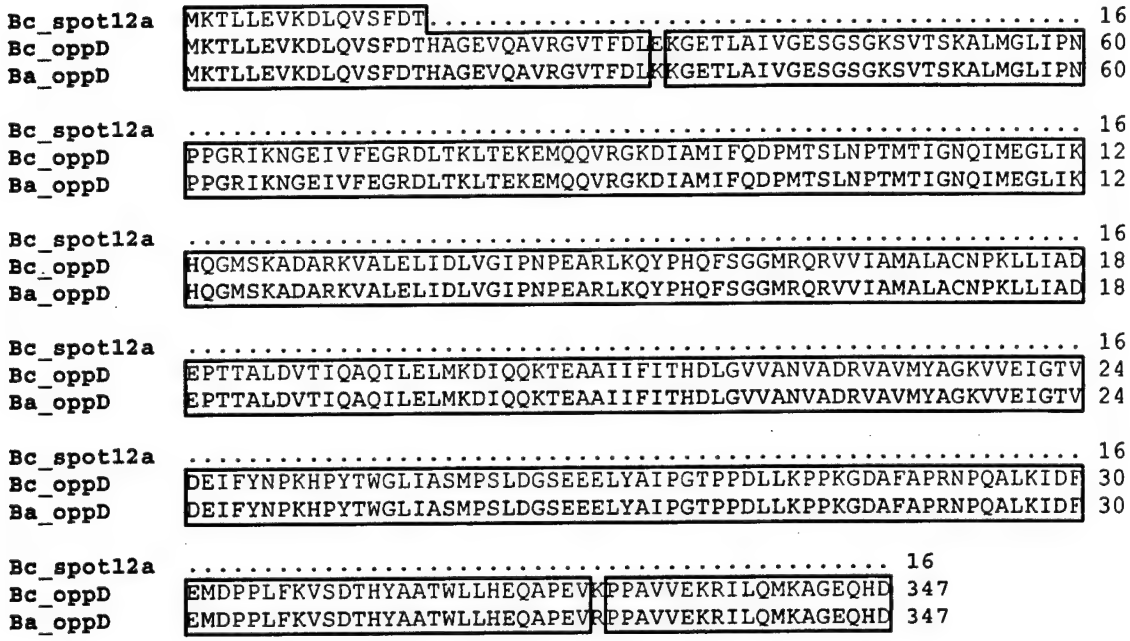

Figure 17. *Oligopeptide ABC transporter. Bc_spot 12a denotes the sequence derived from Edman degradation of spot 12a from a 2D gel. Bc_oppD is the amino acid sequence of oligopeptide ABC transporter found in the B. cereus database. Ba_oppD is the amino acid sequence of oligopeptide ABC transporter found in the B. anthracis database.*

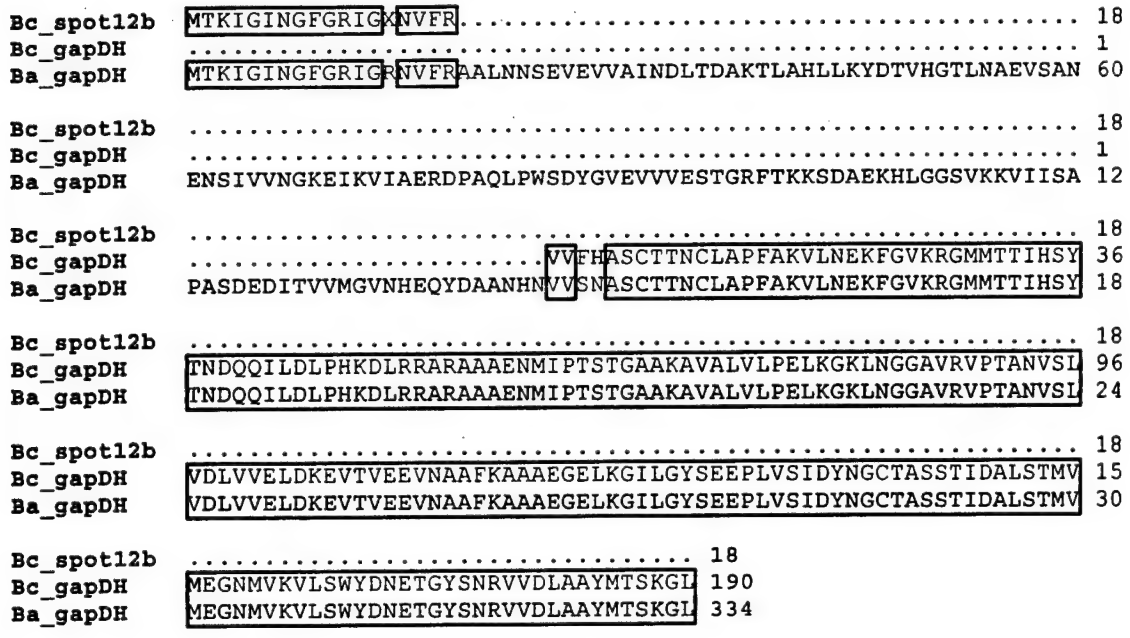

Figure 18. *Glyceraldehyde 3-phosphate dehydrogenase. Bc_spot 12b denotes the sequence derived from Edman degradation of spot 12b from a 2D gel. Bc_gapDH is the amino acid sequence of glyceraldehyde 3-phosphate dehydrogenase found in the B. cereus database. Ba_gapDH is the amino acid sequence of glyceraldehyde 3-phosphate dehydrogenase found in the B. anthracis database.*

Figure 19. *Pyruvate dehydrogenase (E1) beta subunit. Bc_spot 12b denotes the sequence derived from Edman degradation of spot 12b from a 2D gel. Bc_gapDH is the amino acid sequence of glyceraldehyde 3-phosphate dehydrogenase found in the B. cereus database. The n-terminus of this protein falls within a gap in the database. Ba_gapDH is the amino acid sequence of glyceraldehyde 3-phosphate dehydrogenase found in the B. anthracis database.*

dehydrogenase multienzyme complex catalyzes the reaction that produces acetyl coenzyme A from pyruvate, yielding NADH [40]. This is a crucial step between glycolysis and the tricarboxylic acid cycle, where acetyl coenzyme A can further be oxidized to generate energy and intermediates for anabolic reactions [40]. The beta subunit of E1 is part of a complex of 2 beta and 2 alpha chains within pyruvate dehydrogenase [25]. It has also been found present in the soluble fraction of sporulating cells of *B. subtilis* where it functions along with the E2 subunits as check points for stage II-III of sporulation [25]. Therefore, it is likely that E1 would be contained within the spore as part of the main metabolic pathway in order to produce the products needed for germination. It is also interesting to note that it is specifically involved in monitoring sporulation and hence it is not surprising that it would be found within the spore.

The amino acid sequence derived from spot 14 was identified as the ribosomal protein S2 (rpS2). The sequence was a good match for rpS2 in *B. anthracis* and *Streptococcus pneumoniae* (Table 1 and Figure 20). The calculated molecular weight and pI of rpS2 in *B. anthracis* also fell within the range of molecular weight and pI of the excised spot (Table 2). The N-terminus of the corresponding *B. cereus* sequence was missing due to the presence of a gap in the genome data. In prokaryotes the ribosome is composed of two domains, 50S and 30S. The 30S contains the subunits S1 through S21, while the 50S contains subunits L1 through L34. Both of these domains are necessary for protein synthesis [28]. It is not surprising then for this portion of the ribosome to be found within the spore since protein synthesis is critical for germination of the spore. In addition, it is well known that spores contain numerous ribosomes [41].

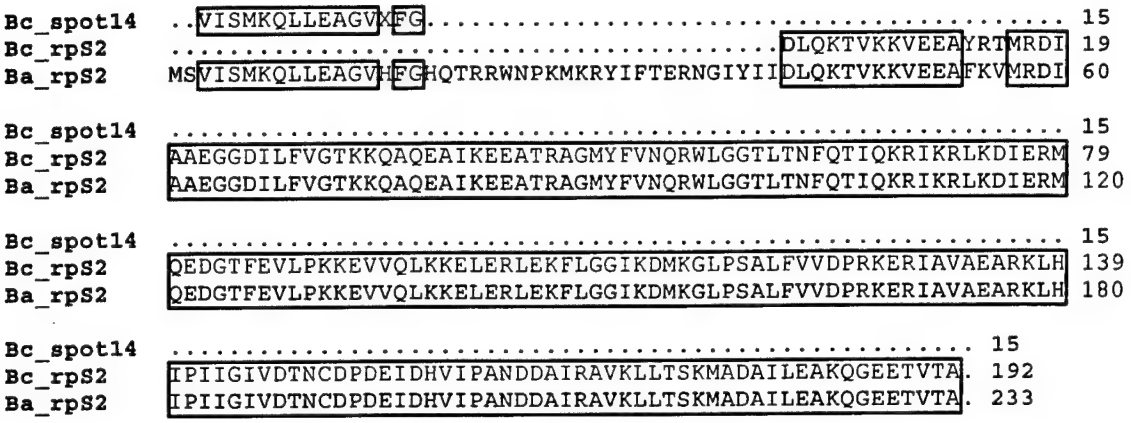

Figure 20. *Ribosomal protein S2. Bc_spot 14 denotes the sequence derived from Edman degradation of spot 14 from a 2D gel. Bc_rpS2 is the amino acid sequence of ribosomal protein S2 found in the B. cereus database. The n-terminus of this protein falls within a gap in the database. Ba_rpS2 is the amino acid sequence of ribosomal protein S2 found in the B. anthracis database.*

The amino acid sequence derived from spot 15 was identified as camelysin 1 (cam1). The sequence matched with camelysin in *B. cereus*, a spore coat protein in *B. thuringiensis* and a hypothetical protein in *B. anthracis* (Table 1 and Figure 21). The ranges of molecular weight and pI for the excised spot contained the calculated value for cam1 in *B. cereus* and *B. anthracis* (Table 2). There are two camelysins with differing pI values that matched with the sequence from spot 15. These two subtypes were labeled camelysin 1 with a pI of 4.3 and camelysin 2 with a pI of 5.2. Since spot 15 had a pI value closest to 4.3 it was labeled camelysin 1. The protein sequence derived from spot 15 was also missing the N-terminus indicating that it contained a leader sequence that was removed during processing (Figure 21). This result is consistent with earlier findings in a previous study of camelysin from *B. cereus* [42]. Camelysin is a novel membrane proteinase of *B. cereus* that has been shown to

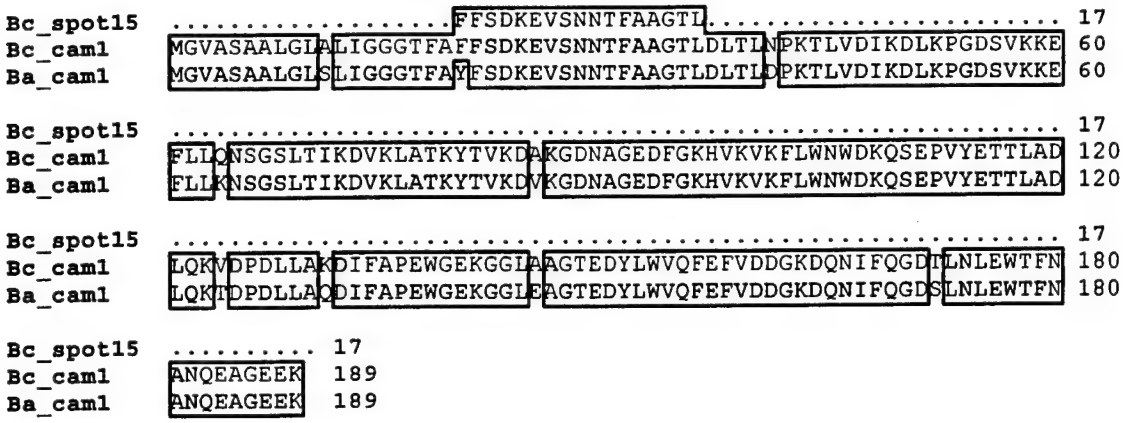

Figure 21. *Camelysin 1. Bc_spot 15 denotes the sequence derived from Edman degradation of spot 15 from a 2D gel. Bc_cam1 is the amino acid sequence of camelysin 1 found in the B. cereus database. Ba_cam1 is the corresponding sequence found in the B. anthracis database.*

break down casein as well as collagen, globin, ovalbumin, and insulin [42]. This substrate range makes it important in the pathogenicity of *B. cereus*. Camelysin interacts with proteins of the blood coagulation cascade and could facilitate the penetration of fibrin clots and of the extracellular matrix during bacterial infection [42]. However, the function of camelysin in the spore is unknown.

The amino acid sequence derived from spot 16 was identified as manganese dependant superoxide dismutase (sodMn). The sequence matched with sodMn in *B. cereus*, *B. anthracis* and *Staphylococcus epidermidis* (Table 1 and Figure 22), although the *B. cereus* sequence was missing the first nine N-terminal residues due to a gap in the genome data. The ranges of molecular weight and pI of the excised spot were slightly lighter and less acidic than sodMn in *B. anthracis* (Table 2). All aerobes and facultative aerobes require superoxide dismutase (SOD) in order to remove superoxide (O_2^-) which is toxic to the cell [27]. SOD combines two molecules of superoxide to form one molecule of hydrogen peroxide and one molecule of oxygen , and all SODs contain a metal cofactor such as Mn^{2+} [27]. It would be important for a spore to contain SODs for protection from the toxic effects of superoxides generated during germination.

```
Bc_spot16   MAKHELPNLPYAYDALEP..................................... 18
Bc_sodMn    .........PYAYDALEPHFDKETMNIHHTKHHNTYITNLNAALEGHAELADKSVEELVA 51
Ba_sodMn    MAKHELPNLPYAYDALEPHFDKETMNIHHTKHHNTYITNLNAALEGHAELADKSVEELVA 60

Bc_spot16   ..................................................... 18
Bc_sodMn    NLNEVPEAIRTAVRNNGGGHANHTFFWTILSPNGGGQPVGELATAIEAKFGSFDAFKEEF 111
Ba_sodMn    NLNEVPEAIRTAVRNNGGGHANHTFFWTILSPNGGGQPVGELATAIEAKFGSFDAFKEEF 120

Bc_spot16   ..................................................... 18
Bc_sodMn    AKAGATRFGSGWAWLVVNNGELEVTSTPNQDSPLTEGKTPVIGLDVWEHAYYLNYQNRRP 171
Ba_sodMn    AKAGATRFGSGWAWLVVNNGELEVTSTPNQDSPLTEGKTPVIGLDVWEHAYYLNYQNRRP 180

Bc_spot16   ..................... 18
Bc_sodMn    DYIGAFWNVVDWNAAEK..... 188
Ba_sodMn    DYIGAFWNVVDWNAAEKRYQEAK 203
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Figure 22. *Superoxide Dismutase Mn. Bc_spot 16 denotes the sequence derived from Edman degeradtion of spot 16 from a 2D gel. Bc_sodMn is the amino acid sequence of superoxide dismutase Mn found in the B. cereus database. Both the n-terminus and the c-terminus fell within gaps in the database. Ba_sodMn is the amino acid sequence of superoxide dismutase Mn found in the B. anthracis database.*

The sequence derived from spot 17 was identified as the ribosomal protein L6 (rpL6). The sequence matched with rpL6 of *B. anthracis* and *Geobacillus stearothermophilus* (Table 1 and Figure 23). The entire sequence of rpL6 was not present in the *B. cereus* database, and presumably fell within a gap in the data. The ranges of molecular weight and pI for the excised spot contain the calculated values of molecular weight and pI for rpL6 in *B. anthracis* (Table 2). As for ribosomal protein S2 above, the presence of ribosomal protein L6 in the spore is not unexpected.

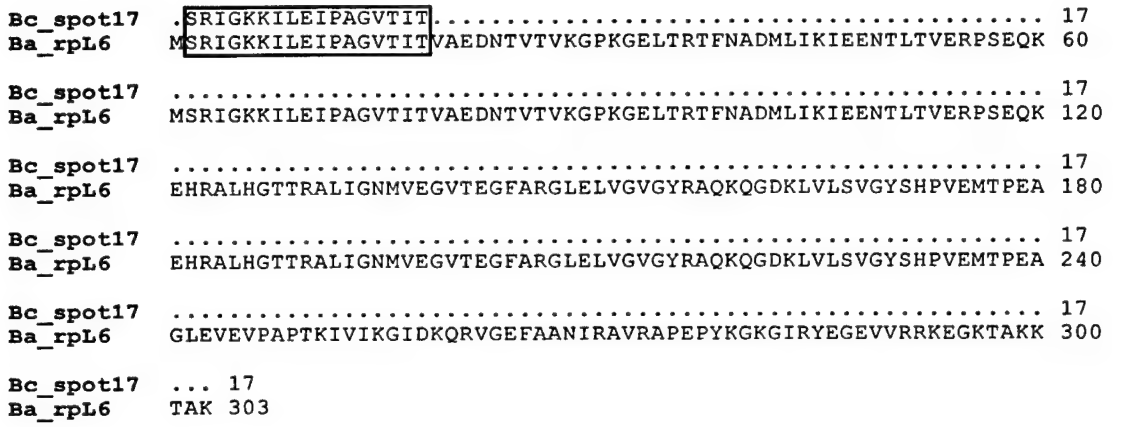

Figure 23. *Ribosomal Protein L6. Bc_spot 17 denotes the sequence derived from Edman degradation of spot 17 from a 2D gel. Ba_rpL6 is the amino acid sequence of ribosomal protein L6 found in the B. anthracis database.*

The amino acid sequence derived from spot 18a was identified as a nitroreductase family protein. This sequence matched with a nitroreductase family protein in *B. anthracis* and *Clostridium acetobutylicum* (Table 1 and Figure 24). In the *B. cereus* database the sequence matched with a hypothetical cytosolic protein (Table 1). The range of molecular weight of the excised spot was slightly lower than the calculated weights of nitroreductase proteins in *B. cereus* and *B. anthracis* (Table 2), but the range of pI of the excised spot contains the calculated pI values of nitroreductase proteins in *B. cereus* and *B. anthracis* (Table 2). Members of the nitroreductase family are involved in the reduction of nitrogen containing compounds such as nitrated polycyclic aromatic hydrocarbons and nitrated pyrenes [43]. They utilize FMN as a cofactor and are often found to be homodimers. Nitroreductase forms a complex with NADPH and catalyzes the reduction of a variety of nitroaromatic compounds to metabolites which are highly toxic, mutagenic, or carcinogenic (Bryant 1991). A recently characterised nitroreductase, the *B. amyloliquifaciens* ywrO gene product, was capable of

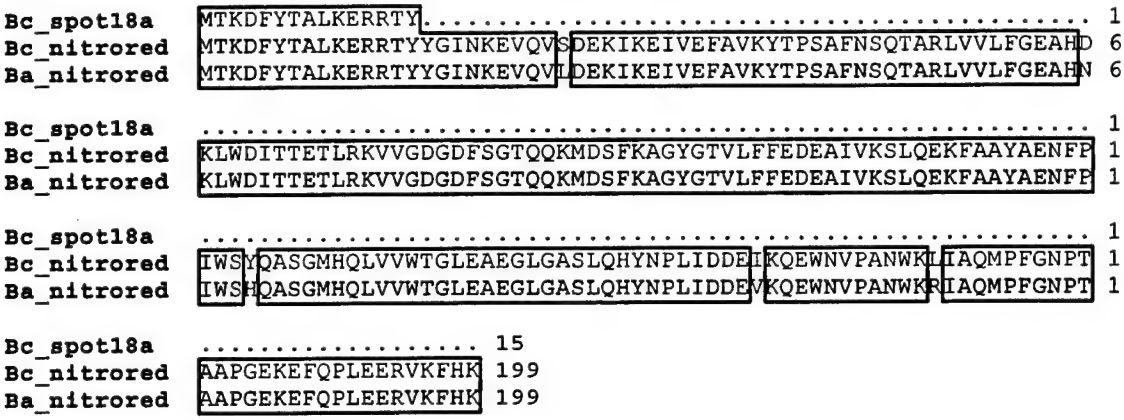

Figure 24. *Nitroreductase family protein. Bc_spot 18a denotes the sequence derived from Edman degradation of spot 18a from a 2D gel. Bc_nitrored is the amino acid sequence of a nitroreductase family protein found in the B. cereus database. Ba_nitrored is the amino acid sequence of a nitroreductase family protein found in the B. anthracis database.*

activating the prodrug 5-aziridinyl-2,4,-dinitrobenzamidine (CB1954) [44]. Such activity suggests that the presence of a nitroreductase in *Bacillus* spores might be exploited for therapeutic applications.

The second amino acid sequence derived from spot 18 (designated 18b) was identified as stage V sporulation protein T (s5spT). This sequence matched with the sequence of s5spT in *B. cereus* and *Clostridium acetobutylicum* (Table 1 and Figure 25). The sequence matched with a hypothetical protein in the *B. anthracis* database (Table 1 and Figure 25). The range of molecular weight and pI of the excised spot were slightly smaller and less acidic than the calculated values of molecular weight and pI for s5spT in *B. cereus* and *B. anthracis* (Table 2). There are seven stages of sporulation from the vegetative stage (Stage 0) to the free spore stage (Stage VII) [27]. During Stage V of this process the spore coat layers are formed and S5spT is a transcription regulator that is required for spore cortex synthesis [28]. During the stationary phase of cell growth, when the viable count remains steady s5spT acts as a sporulation inhibitor [28]. This check point is important to ensure that sporulation only occurs when absolutely necessary.

```
Bc_spot18b   MKATGIVRRIDDLGRVV..................................................  17
Bc_s5spT     MKATGIVRRIDDLGRVVIPKEIRRTLRIREGDPLEIFVDRDGEVILKKYSPISELGDFAK  60
Ba_s5spT     MKATGIVRRIDDLGRVVIPKEIRRTLRIREGDPLEIFVDRDGEVILKKYSPISELGDFAK  60

Bc_spot18b   ..................................................................  17
Bc_s5spT     EYADALYDSLGHNVLVCDRDSIIAVSGVSKKEYLNKSVGDLIEKTMEERKSVIMTDESDI  12
Ba_s5spT     EYAEALYDSLGHNVLVCDRDSIIAVSGVSKKEYLNKSVGDLIEKTMEERKSVIMTDESDV  12

Bc_spot18b   ..................................................................  17
Bc_s5spT     SIIDGVTEKVHSYTVGPIVANGDPIGAVIIFSKEAIISEIEHKAVNTAASFLAKQMEQ  178
Ba_s5spT     SIIDGVTEKVHSYTVGPIVANGDPIGAVIIFSKEAIISEIEHKAVNTAASFLAKQMEQ  178
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Figure 25. *Stage V sporulation protein T. Bc_spot 18b denotes the sequence derived from spot 18b on the 2D gel. Bc_s5spT is the amino acid sequence of stage V sporulation protein found in the B. cereus database. Ba_s5spT is the amino acid sequence of stage V sporulation protein T found in the B. anthracis database.*

The amino acid sequence derived from spot 19 was identified as alkyl hydroperoxide reductase subunit C (ahpC). The sequence matched with ahpC of *B. cereus*, *B. anthracis*, and *Xanthomomas axonopodis* (Table 1 and Figure 26). The range of molecular weight and pI was again somewhat lower and less acidic than the calculated values for ahpC in *B. cereus* and *B. anthracis* (Table 2). AhpC is the catalytic subunit responsible for alkyl peroxide metabolism [45]. In *Xanthomonas* alkyl hydroperoxide reductase genes (*ahpC* and *ahpF*) and a novel family of organic peroxide resistance genes (*ohr*) have been identified, and are involved in organic peroxide protection [45]. Alkyl hydroperoxide reductase is the best characterized microbial enzyme involved in organic peroxide metabolism and consists of a catalytic 22-kDa C subunit (AhpC) and a reductase 52-kDa F subunit (AhpF) [45]. Inactivation of ahpC in various bacterial mutants results in increased sensitivity to organic peroxide killing and to spontaneous mutagenesis. Therefore it is possible that ahpC would be contained within the spore for protection from harsh environments.

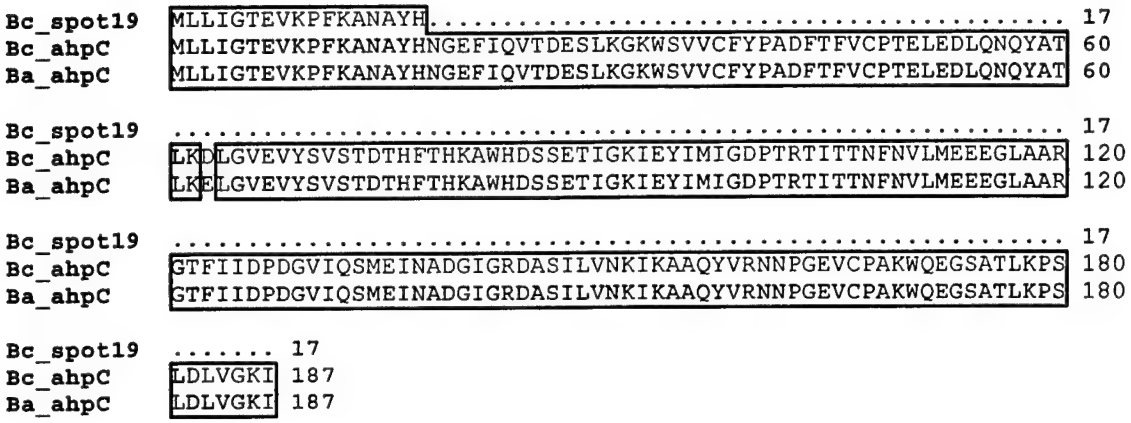

Figure 26. *Alkyl hydroperoxide reductase subunit C. Bc_spot 19 denotes the sequence derived from Edman degradation of spot 19 from a 2D gel. Bc_ahpC is the amino acid sequence of alkyl hydroperoxide reductase subunit C found in the B. cereus database. Ba_ahpC is the amino acid sequence of alkyl hydroperoxide reductase subunit C found in the B. anthracis database.*

The amino acid sequence derived from spot 20 was identified as camelysin 2 (cam2). The sequence matched with a spore coat protein in *B. cereus* and *B. thuringiensis* and a hypothetical protein in *B. anthracis* (Table 1 and Figure 27). The ranges of molecular weight and pI for the excised spot were slightly lower and less acidic than the calculated value for cam2 in *B. cereus* and *B. anthracis* (Table 2). As mentioned above there are two camelysins with differing pI values in *B. cereus*. Of these, camelysin 2 with a pI of 5.2 was the best match for the sequence in spot 20. The protein sequence derived from spot 20 was also missing the N-terminus indicating that it contained a leader sequence that was removed during processing (Figure 27).

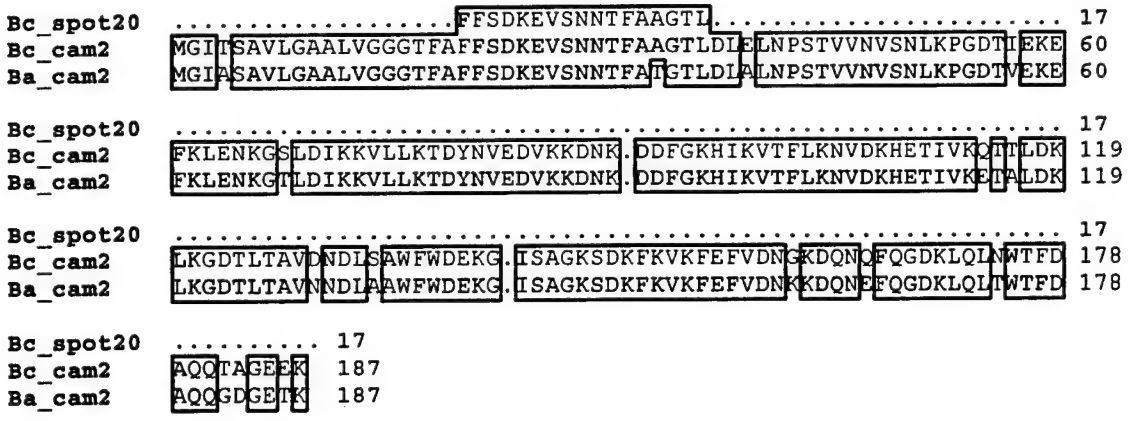

Figure 27. *Camelysin 2. Bc_spot 20 denotes the sequence derived from spot 20 from a 2D gel. Bc_cam2 is the amino acid sequence of camelysin 2 found in the B. cereus database. Ba_cam2 is the amino acid sequence of camelysin 2 found in the B. anthracis database.*

Conclusions

From the preceding data it can be confirmed that *B. cereus* is an excellent model for studies on *B. anthracis*. Each sequence that was analyzed matched very well with a specific protein in the *B. cereus* and *B. anthracis* databases. The sequences for the proteins found in the *B. cereus* database all show extremely high identity with the sequences of the same protein in the *B. anthracis* database.

It is also important to consider the possible anti-BW applications that can be drawn from this study. Knowledge of the proteins contained within the bacterial spore is the first step in the design of specific inhibitors which either kill the spore or prevent its germination. Preventing the spore from germinating would reduce the pathogenicity of the bacteria, as it is the vegetative cell that causes the disease.

It should be noted that no structural proteins were identified in this study, although undoubtedly they are part of the spore. It is likely that these proteins were either not solubilized by the buffer or tightly bound to the insoluble material. Since any such material was sedimented out by centrifugation prior to focusing, such proteins would not be found in our gels.

The results of this study have opened the door to further identification of the proteins contained within the *B. cereus* spore. Only 20 spots of the approximate 153 were analyzed, leaving plenty of room for further exploration. The methods devised in this study also provide a basis for future studies on the presence of different proteins expressed by the spore under various conditions, such as treatment with antibiotics, or nutrient limitation. In addition comparative analysis of *B. cereus* and *B. anthracis* spot patterns should highlight any potential differences in the constituents of the respective spores.

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List of symbols/abbreviations/acronyms/initialisms

DND	Department of National Defence
DTT	dithiothreitol
SDS	sodium dodecyl sulfate
PAGE	polyacrylamide gel electrophoresis
CHAPS	([3-chloamidopropyl]-dimethyammonio)-1-propane-sulfonamide
IPG	immobilised pH gradients
PVDF	polyvinylidene difluoride
IEF	isoelectric focussing
APS	ammonium persulfate
2D	two-dimensional
DRA	Defence Research Assistant
CBDS	Chemical and Biological Defence Systems
DRDC	Defence Research and Development Canada
EF-G	Translation elongation factor
Hsp70	Heat shock protein 70
OpdB	Oligopeptidase B
SucDH	Succinate dehydrogenase
Cpn60	60 kDa chaperonin
P5cDH	Pyrroline-5-carboxylate dehydrogenase
ALDH	Aldehyde dehydrogenase
E2	Dihydrolipoamide acetyltransferase
ATPsynt-a	ATP synthase alpha subunit

E3	Dihydrolipoamide dehydrogenase
ATPsynt-b	ATP synthase beta subunit
Ala-rac	Alanine racemase
LeucDH	Leucine dehydrogenase
OppD	Oligopeptide ABC transporter
GapDH	Glyceraldehyde 3-phosphate dehydrogenase
E1	Pyruvate dehydrogenase
RpS2	Ribosomal protein S2
Cam1	Camelysin 1
SodMn	Superoxide dismutase (Mn)
S5spT	Stage V sporulation protein T
AhpC	Alkyl hydroperoxide reductase subunit C
Cam2	Camelysin 2

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Spores were harvested from *Bacillus cereus* 14579 grown in casitone-yeast medium for 4 days at 30°C. Two-dimensional gel electrophoresis was performed on solubilized spore protein and twenty of the most abundant spots were analyzed by N-terminal amino acid sequencing. The identities of the N-terminal sequences obtained were determined by homology searching of the Genbank non-redundant database, and the *B. anthracis* and *B. cereus* genome projects. All of the identified proteins were plausible spore components, and included chaperonins, sporulation regulators, ribosomal proteins, proteases, and metabolic enzymes involved in energy production, radical detoxification, and germination. The conservation and extremely high identity of the identified proteins in both the *B. cereus* and *B. anthracis* genome databases confirmed the applicability of *B. cereus* spores as a surrogate model for the study of *B. anthracis* spores.

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Bacillus cereus, spores, two-dimensional electrophoresis, N-terminal sequencing

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